Optimization of formulated artificial diets with the addition of sterols and cryoprotectants for effective rearing and fitness of *Eldana saccharina* Walker (Lepidoptera: Pyralidae)

by

Nomalizo Carol Ngomane

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Supervisor: Professor Des E. Conlong

Co-supervisors: Professor John S. Terblanche and Dr Elsje Pieterse

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Summary

Eldana saccharina is amongst the most economically damaging pests in South African sugarcane. Many attempts have been made to control the pest. The Sterile Insect Technique (SIT) as part of an Integrated Pest Management (IPM) program against *E. saccharina*, offers great potential to reduce pest damage to below the economic injury level. Since the success of SIT depends upon the production of high quality, competitive sterile male insects for field releases, it follows that mass-rearing of *E. saccharina* on artificial diet is a principal step in the process. To this end, three separate trials were conducted to study the nutritional requirements and formulate better and more economical diets for mass rearing *E. saccharina*. The diet currently used to rear this species was developed from a previously published diet for *Ostrinia nubilalis*, which, even though it was much more efficient and cost effective than previous diets developed for *E. saccharina*, it specifically did not take into account the actual nutrient requirements needed for optimal development of *E. saccharina*. Four artificial diets, based on the following were formulated using the carcass milling technique: the first, formulated according to the minimum specification of a summary of literature diets proven to be effective at rearing *E. saccharina* (MS); and the other three based on the ideal amino acid composition and profile (IAAP) of the second (IAAP2), third/fourth (IAAP3/4) and fifth/sixth (IAAP5/6) instar larvae. The current diet used at SASRI was used as the control diet (ECBMOD).

Survival was significantly high on all diet formulations with more than 92 % of inoculated neonate *E. saccharina* life stages surviving at day 20 and more than 95 % surviving up till harvest (Day 27). The life stages developed fastest in the IAAP3/4 and MS diets (25 % and 17 % prepupae and pupae produced at day 20, respectively) compared to life stages from the remaining carcass milling diets and the control diet. Within dietary formulations female pupae were significantly heavier (0.1908 g) than male pupae (0.1138 g). Male and female pupal weights were not significantly influenced by the carcass milling diets, as the control diet produced heavier male and female pupae (0.1204 g and 0.2085 g, respectively) compared to them. Adult emergence from pupae was significantly highest (98 %) for the MS diet, followed by the IAAP3/4 (97 %) and control diet (96 %). The sex ratio of adults emerging from pupae harvested from the different diet formulations was close to 1. There were no significant differences observed in male chill coma recovery time (recovery time: 267.60 s) but females from the IAAP5/6 diet recovered the fastest from chill coma treatment. Males from the IAAP5/6 diet mated with significantly more females (6 different females) than those of the remaining diets, who mated with an average of 4 different females. Although not significantly different from the control diet, females from the MS diet mated with more males (3 different males) than those of the remaining carcass milling diets, who mated with an average of 1 male. Females from all diet formulations produced more than 870 eggs that were more than 90 % fertile. The physical properties (pH: 4.79, moisture content: 81.43 % and water activity: 0.92 a_w) of the diets were not significantly different, and maintained the quality and stability of the diets produced, ensuring optimal growth and development of *E. saccharina* throughout the trial duration.

Due to the faster larval development of *E. saccharina* reared on the MS diet, further improvements to this diet were investigated through the inclusion of sterols (cholesterol (C) and stigmasterol (S)), which have been shown to be essential to insect growth and have improved insect performance when fed to them in artificial diets. The larval development period was significantly shortened in the sterol diets, compared to the MS diet without sterols added, irrespective of the type and concentration of sterol added. Larvae that developed fastest, determined by highest percentage of pupae 20 days after diet inoculation, was recorded on the MS (1.0gS) diet (72 %) followed by the MS (0.2gC: 0.2gS) diet (70 %). Pupal weight was increased on females that fed on the MS (0.1gC), MS (0.1gS) and MS (0.2gC:0.2gS) (0.2143 g, 0.2271 g and 0.2252 g, respectively) compared to those of the MS diet without sterols added (0.1864 g). To improve the cold tolerance of this insect to make it more fit for the environmental conditions into which it would be field released, the inclusion of cryoprotectants (i.e. L-proline (P) and trehalose (T)) into the MS diet was also investigated. Pupal weight increased in males (0.1295 g) that fed on the MS (0.2gP:0.2gT) diet and the chill coma

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recovery time of male and female moths (204.00 s and 259.20 s, respectively) was reduced on *E. saccharina* reared on this same diet, compared to that of the MS diet without cryoprotectants added (253.20 s and 306.60 s, respectively). The addition of cryoprotectants into the MS diet did not improve fertility of chill coma exposed female moths, but instead it severely reduced fertility to less than 44 %, compared to females not exposed to the chilling treatment whose eggs were on average 84 % fertile.

The carcass milling technique proved to be effective at developing superior diets (i.e. the MS and IAAP3/4 diets) than the *Ostrinia* based diet, and their qualities as a good food source were even more improved when sterols and cryoprotectants were added (particularly in the MS diet) as supplements. The findings of this study demonstrated that the MS diet incorporated with the lower concentration of the sterol mix (0.2gC:0.2gS) and the cryoprotectant mix (0.2gP:0.2gT) can result in a positive impact on *E. saccharina's* life history traits, indicating that this species can be effectively mass reared with a significant reduction in rearing time and resultant costs for the SIT program. The MS diet formulation including the sterol mix (0.2gC:0.2gS) is the preferred choice to replace the current diet used to rear *E. saccharina* at SASRI, as it reduced the larval growth period dramatically by 60 % compared to the other diets in this study, including those incorporating cryoprotectants, without having any negative effects on key quality parameters of *E. saccharina*.

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Opsomming

Eldana saccharina is een van die mees skadelikste plae in suikerriet in Suid-Afrika. Daar is baie pogings aangewend om die plaag te bestry. Die Steriele Insek Tegniek (SIT) as onderdeel van 'n geïntegreerde plaagbeheer (IPM) teen *E. saccharina*, bied 'n groot potensiaal om plaagskade tot onder die ekonomiese beseringsvlak te verminder. Aangesien die sukses van SIT afhang van die produksie van kompeterende steriele manlike insekte van hoë gehalte vir veldvrystelling, volg dit dat die grootmaak van *E. saccharina* op kunsmatige dieët 'n belangrike stap in die proses is. Vir hierdie doel is drie afsonderlike proewe gedoen om die voedingsbehoeftes te bestudeer en beter en meer ekonomiese diëte vir die grootmaak van *E. saccharina* te formuleer. Die dieët wat tans gebruik word om hierdie spesie groot te maak, is ontwikkel uit 'n voorheen gepubliseerde dieët vir *Ostrinia nubilalis*, wat, hoewel dit baie meer doeltreffend en koste-effektief was as vorige diëte wat vir *E. saccharina* ontwikkel is, het dit nie spesifiek die werklike voedingsstowwe in ag geneem wat benodig word vir optimale ontwikkeling van *E. saccharina*. Vier kunsmatige diëte, gebaseer op die volgende, is geformuleer met behulp van die karkasmaaltegniek: die eerste, geformuleer volgens die minimum spesifikasie van 'n opsomming van literatuur diëte wat bewys word dat dit effektief is om *E. saccharina* (MS) groot te maak; en die ander drie gebaseer op die ideale aminosuursamestelling en -profiel (IAAP) van die tweede (IAAP2), derde/vierde (IAAP3/4) en vyfde/sesde (IAAP5/6) stadium larwes. Die huidige dieët wat by SASRI gebruik is, is as die beheerdieët (ECBMOD) gebruik.

Die oorlewing was beduidend hoog op alle dieëtformulerings, met meer as 92 % geëntde pasgebore *E. saccharina*-lewensfases wat op dag 20 oorleef het en meer as 95 % tot die oes oorleef (Dag 27). Die lewensfases het die vinnigste ontwikkel in die IAAP3/4- en MS-diëte (25 % en 17 % prepupae en papies wat onderskeidelik op dag 20 geproduseer is) in vergelyking met lewensfases van die oorblywende karkasmaal-diëte en die kontroledieët. Binne dieëtformulerings was vroulike papies aansienlik swaarder (0.1908 g) as manlike papies (0.1138 g). Die gewig van die manlike en die vroulike papie is nie beduidend beïnvloed deur die karkasmaal-diëte nie, aangesien die kontroledieët swaarder manlike en vroulike papies (onderskeidelik 0.1204 g en 0.2085 g) opgelewer het. Volwassenes se opkoms van papies was beduidend die hoogste (98 %) vir die MS-dieët, gevolg deur die IAAP3/4 (97 %) en kontrole-dieët (96 %). Die geslagsverhouding van volwassenes wat voortspruit uit papies wat uit die verskillende dieëtformulerings geoes is, was ongeveer 1. Daar is geen beduidende verskille waargeneem in hersteltyd vir koue koma (hersteltyd: 267,60 s) nie, maar vroue uit die IAAP5/6-dieët het die vinnigste herstel koue koma behandeling. Mans van die IAAP5/6-dieët het gepaar met aansienlik meer wyfies (6 verskillende wyfies) as dié van die oorblywende diëte, wat gemiddeld met 4 verskillende wyfies gepaar het. Alhoewel dit nie beduidend van die kontrole-dieët verskil nie, het vrouens uit die MS-dieët gepaar met meer mans (3 verskillende mans) as dié van die oorblywende karkas-maal-diëte, wat gemiddeld met 1 mannetjie gepaar is. Wyfies uit alle dieëtformulerings het meer as 870 eiers geproduseer wat meer as 90 % vrugbaar was. Die fisiese eienskappe (pH: 4,79, voginhoud: 81,43 % en wateraktiwiteit: 0,92 a_w) van die diëte was nie beduidend verskillend nie en het die kwaliteit en stabiliteit van die geproduseerde diëte gehandhaaf, wat die optimale groei en ontwikkeling van *E. saccharina* gedurende die proeftydperk verseker het.

As gevolg van die vinniger larwale ontwikkeling van *E. saccharina* wat op die MS-dieët geteel is, is verdere verbeterings aan hierdie dieët ondersoek deur die insluiting van sterole (cholesterol (C) en stigmasterol (S)), wat bewys is dat dit noodsaaklik is vir die groei van insekte, en het insekprestasie verbeter as dit in kunsmatige diëte aan hulle gevoer word. Die ontwikkelingsperiode vir larwes is in die steroldiëte aansienlik verkort, vergelyke met die MS-dieët sonder dat sterole bygevoeg is, ongeag die tipe en konsentrasie sterol wat bygevoeg is. Larwes wat die vinnigste ontwikkel het, bepaal deur die hoogste persentasie papies 20 dae na dieëtinenting, is aangeteken op die MS (1.0gS) dieët (72 %), gevolg deur die MS (0.2gC: 0.2gS) dieët (70 %). Die gewig van die papies is verhoog by vroue wat grootgemaak is op MS (0.1gC), MS (0.1gS) en MS (0.2gC: 0.2gS) (onderskeidelik 0.2143 g, 0.2271 g en 0.2252 g) vergelyk met dié van die MS-dieët sonder sterole bygevoeg (0.1864 g). Die insluiting van kriobeskermingsmiddels (dws L-proline (P) en trehalose (T))

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in die MS-dieët is ook ondersoek om die kouetoleransie van hierdie insek meer geskik te maak vir die omgewingstoestande waarin dit in die veld vrygestel word. Die gewig van die papies het toegeneem by mans (0.1295 g) wat die MS-dieët (0.2gP: 0.2gT) gevoer is.Die hersteltyd van koue koma van manlike en vroulike motte (onderskeidelik 204,00 en 259,20 s) is verminder op *E. saccharina* wat grootgemaak is op dieselfde dieët, in vergelyking met die van die MS-dieët sonder dat kriobeskermingsmiddels bygevoeg is (onderskeidelik 253,20 en 306,60 s). Die toevoeging van kriobeskermingsmiddels in die MS-dieët het die vrugbaarheid van vroulike motte wat aan koue koma blootgestel is, nie verbeter nie, maar dit verminder vrugbaarheid tot minder as 44 %, vergeleke met wyfies wat nie blootgestel is aan die koue behandeling nie, waarvan die eiers gemiddeld 84 % vrugbaar was.

Die karkasmaaltegniek blyk effektief te wees vir die ontwikkeling van superieure diëte (dws die MS- en IAAP3/4-diëte) as die *Ostrinia*-dieët, en die eienskappe daarvan as 'n goeie voedselbron is nog beter as sterole en kriobeskermingsmiddels bygevoeg word (veral in die MS-dieët) as aanvullings. Die bevindinge van hierdie studie het getoon dat die MS-dieët wat saam met die laer konsentrasie van die sterolmengsel (0.2gC: 0.2gS) en die kriobeskermingsmengsel (0.2gP: 0.2gT), 'n positiewe uitwerking op *E. saccharina* se lewensgeskiedenis kan hê, wat aandui dat hierdie spesie effektief grootgemaak kan word met 'n aansienlike vermindering van die opvoedingstyd en gevolglike koste vir die SIT-program. Die MS-dieëtformulering, insluitend die sterolmengsel (0.2gC: 0.2gS), is die voorkeur keuse om die huidige dieët wat gebruik word om *E. saccharina* by SASRI te verhoog, te vervang, aangesien dit die larwagroeiperiode dramaties verminder het met 60 % in vergelyking met die ander diëte in hierdie studie, insluitend diegene wat kriobeskermingsmiddels bevat, sonder om negatiewe effekte op die sleutel kwaliteits parameters van *E. saccharina* te hê.

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Chapter 1. Introduction and Literature Review

1.1 *Eldana saccharina*: A pest in South African sugarcane

Eldana saccharina Walker (Lepidoptera: Pyralidae) has currently spread throughout the South African sugarcane industry, reaching key pest status in the whole region (Conlong, 1990; Horton *et al.*, 2002; Potgieter *et al.*, 2013). It has been recorded for over 100 years as a pest of graminaceous crops, wetland sedges and a number of grasses (Poaceae) in various African countries (Conlong, 1994; Chinheya *et al.*, 2009; Walton and Conlong, 2016). This stalk borer was first described in sugarcane by Walker (1865) in Sierra Leone. Since then it has been recorded throughout sub-Saharan Africa including Mozambique in 1903, South Africa in 1928, South African sugarcane in 1939 and Zimbabwean sugarcane in 1998 (Dick, 1945; Carnegie, 1974; Conlong, 1994; Aseffa *et al.*, 2006; Rutherford, 2015).

Figure 1.1 History of *Eldana saccharina* outbreaks in South Africa and Swaziland (From Rutherford, 2015).

After its initial outbreak in 1939 on the Umfolozi flats, *E. saccharina* populations declined in South African sugarcane and it went unnoticed for several decades. However, in the 1970s populations increased to noticeable levels, infesting sugarcane crops in various parts of northern

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KwaZulu-Natal and spreading steadily southwards (Dick, 1945; Carnegie, 1974; Rutherford, 2015, Walton and Conlong, 2016). It is thought that the shift of *E. saccharina* into sugarcane occurred due to the disturbance and destruction of its natural wetland habitats and planting of sugarcane in these habitats (Rutherford, 2015; Mulcahy, 2018). Furthermore, the morphology of the crop to provide cryptic oviposition sites for female moths to lay eggs and to avoid predation from existing natural enemies, enabled the pest to successfully colonise the new crop host (Horton *et al.,* 2002, Conlong *et al.*, 2007).

1.1.1 Biology and damage symptoms

The life cycle of *E. saccharina* consists of eggs, larvae, pupae and moths. The moths are light brown, with a wing span of approximately 30 mm in males to 39 mm in females. When at rest, their wings typically fold backwards across the abdomen (Carnegie, 1974; Mulcahy, 2018). In general, moths emerge shortly after sunset, with males emerging slightly before females (Carnegie, 1974; Rutherford, 2015; Mulcahy, 2018). The moths live for 6 to 15 days, with males having a maximum lifespan of 7 days (Walton and Conlong, 2016). They do not feed but do drink water. Moths mate either on the first or second night after adult emergence, and females sometimes fly for approximately 200 m or more before they oviposit (Walton and Conlong, 2016; Mulcahy, 2018). Once mated, female moths oviposit yellow oval eggs, most within 3 days (Walton and Conlong, 2016). The female cryptically deposits eggs between dead leaf sheaths and mature sugarcane stalks in the lower third of the plant using its prehensile ovipositor (Walton, 2011). Each female can lay about 400 to 600 eggs which hatch after 8 to 10 days depending on the temperature (Rutherford, 2015; Walton and Conlong, 2016; Mulcahy, 2018).

Atkinson (1980), reported on the number of *E. saccharina* larval instars. Males have 5 to 6, and females 6 to 7 larval instars. Larval colour ranges from light brown to dark grey (Mulcahy, 2018). The larva is tough and active and when it encounters predators it wriggles aggressively in backward movement, excreting a brownish liquid from its mouth which deters predation, and spins down from its host plant on a silken thread (Rutherford, 2015). The neonate larva does not enter the sugarcane stalk immediately after hatching, but instead feeds on organic matter on sugarcane stalk surfaces, protected by dead leaf sheaths (Rutherford, 2015; Mulcahy, 2018). After 10 to 15 days, when the larva is strong enough, it penetrates the plant tissue by boring through sugarcane buds, nodes or cracks in the rind (Mulcahy, 2018). It feeds extensively inside the sugarcane stalk, creating tunnels within the stem and pushing frass to the exterior through moth exit holes (Rutherford, 2015; Mulcahy, 2018). There is an association between *E. saccharina* larval feeding and a fungus (*Fusarium* spp.) which causes red discoloration on the damaged plant tissue (McFarlane *et al.*, 2009; Rutherford, 2015).

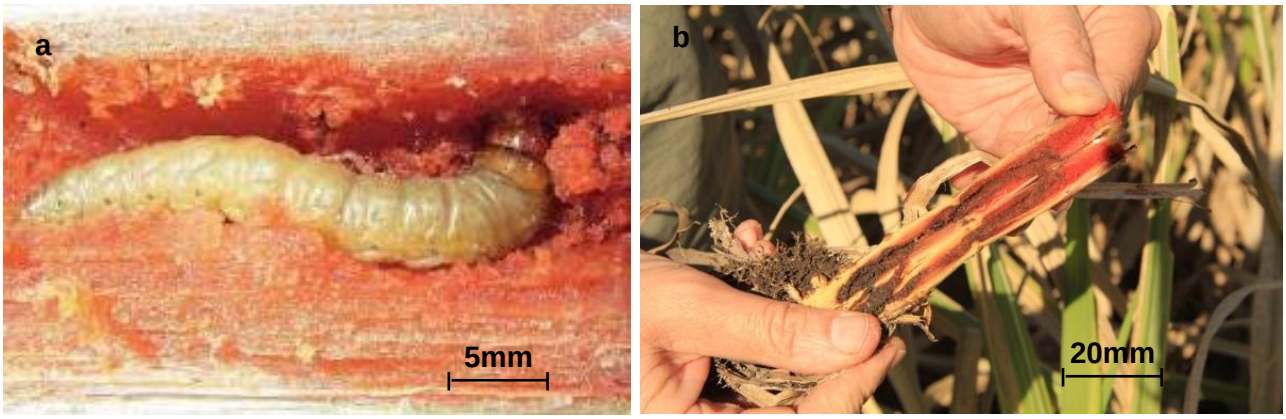

Figure 1.2 (a) *Eldana saccharina* larva inside a sugarcane stalk and (b) the damage caused on the plant tissue. The red colouration caused by *Fusarium* species is clearly seen around the borings (From Rutherford, 2015).

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Larval period varies from 20 days in summer to 60 days in winter (Atkinson, 1980; Rutherford, 2015; Mulcahy, 2018). On maturing, the larva chews an exit window in the rind of sugarcane, and spins a protective cocoon and pupates in it, either in a boring in the stalk, or just outside the exit window behind a leaf sheath. After 7 to 10 days adult moths start to emerge, and mating and breeding continues (Rutherford, 2015; Mulcahy, 2018). Mudavanhu *et al.* (2012) provides a detailed description of *E. saccharina's* mating behaviour in sugarcane and, according to Rutherford (2015), peaks in moth numbers in the field are usually around March/April and September/October, even though generations are multivoltine in nature.

1.1.2 Economic implications

Damage caused by *E. saccharina* larvae, together with the associated fungal infestations, severely reduces sugarcane yield, sucrose content and increases fibre (Chinheya *et al.*, 2009; Rutherford, 2015). Losses in quality are severe, because it attacks the lower half of a mature sugarcane stalk where most of the sugar is stored. In addition, it attacks all varieties of sugarcane (Dick, 1945; Conlong, 2000). A recent estimate of yield losses caused by *E. saccharina* in South African sugarcane adds up to more than ZAR744 million per annum (Rutherford, 2015). Larval feeding can reduce sugarcane yield by 0.1 % for every 1 % of stalks bored (King, 1989; Conlong, 1994) or alternatively, 1 to 4 % of useable sugar can be lost for every 1 % of internodes bored (Rutherford, 2015).

1.1.3 Control methods

Most insect pests have historically been managed by the injudicious use of broad-spectrum, and often persistent insecticides, which are relatively expensive and unsustainable due to pesticide residue issues, increased insecticide resistance of insects and the negative impact insecticides have on human health and the environment (Bloem *et al.*, 2005; Vreysen *et al.*, 2016). To mitigate these challenges, a global consensus proposes that key insect pest management be administered ideally by the concept of Area-wide Integrated Pest Management, which is a strategy applied against an entire target pest population within a defined geographical area (AW-IPM) (Bloem *et al.*, 2005; Vreysen *et al.*, 2016).

1.1.3.1 Integrated Pest Management (IPM)

Integrated Pest Management has been adopted by many agricultural industries as a holistic agro-ecosystem approach to reduce pest populations difficult to control with available conventional technologies (Eze and Echezona, 2012). It does not exclude the use of pesticides but it minimises their use and encourages natural pest control methods including cultural control, mechanical control, habitat management and biological control (Cockburn, 2013; Rutherford, 2015). There is no "silver bullet" for *E. saccharina* pest problems, and it is not practicable to eradicate the pest populations, due to it having a wide range of wild host plants (Atkinson, 1980; Conlong, 1994). Any attempt to do so would be very expensive, unsafe and highly unsuccessful. Therefore, the emphasis of IPM for *E. saccharina* is on suppression and not on eradication (Rutherford, 2015).

In addition to producing sugarcane varieties resistant to *E. saccharina* (Nxumalo and Zhou, 2018), studies conducted by researchers at the South African Sugarcane Research Institute (SASRI) show that *E. saccharina* damage can be greatly reduced if insecticide application is scheduled to overlap with moth peaks when newly hatched scavenging and dispersing neonate larvae are targeted (Leslie, 1997; 2003). They emphasise the importance of applying silicon to improve resistance of sugarcane to *E. saccharina* infestations (Meyer and Keeping, 2005), and propose best management practices integrated into an AW- IPM programme to reduce plant stress, thus reducing the potential of *E. saccharina* damage to an extent that longer cropping cycles become possible (Rutherford, 2015). Although current IPM strategies implemented at SASRI promote lower *E. saccharina* infestations, a species specific and environmentally friendly control tactic known as the

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Sterile Insect Technique offers great potential as a further tool in the IPM toolbox, to reducing pest damage to more tolerable levels (Vreysen *et al.*, 2016).

1.1.3.2 Sterile Insect Technique (SIT)

The SIT is an area-wide insect pest control method targeting an entire insect pest population. It was first introduced by 3 independent researchers, A.S. Serebrovskii, F. L. Vanderplank and E.F. Knipling as a potential insect control technique in the 1930s and 1940s (Klassen, 2005; Walton *et al.*, 2011). The basis of the technique involved mass rearing target pest species, introducing sexual sterility through x-ray or gamma irradiation of pupae or adult males, followed by sustained field releases of sterile male insects in numbers sufficient to obtain appropriate sterile to wild male overflooding ratios. In Lepidoptera SIT, F1 male sterility is aimed for, as this Order of insects is known to be more resistant to irradiation than Diptera (Walton *et al*., 2011). Here, released partially sterile males will mate with wild virgin females and, as a result, the females will lay partially infertile eggs producing fewer offspring (F1 generation), most of whom will be completely sterile and predominately male, thus reducing productivity of that population (Walton *et al.*, 2011; Hofmeyr *et al.*, 2016; Mudavanhu *et al.*, 2016; Vreysen *et al.*, 2016).

Over the past decade, SIT has had successful initiatives targeting a number of major dipteran pest species such as Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae), melon fruit fly, *Bactrocera cucurbitae* Coquillett (Diptera: Tephritidae), New World screwworm, *Cochliomyia hominivorax* Coquerel (Diptera: Calliphoridae) and the tsetse fly, *Glossina austeni* Newstead (Diptera: Glossinidae). Successful lepidopteran programmes have included pink bollworm, *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae), codling moth, *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae), false codling moth, *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae), cactus moth, *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) and the Australian painted apple moth, *Orgyia anartoides* Walker (Lepidoptera: Lymantriidae) (Hofmeyr *et al.*, 2016; Vreysen *et al.*, 2016). All these programs owe their success to being able to mass rear many insects of high quality consistently and at relatively low cost (Leppla *et al.*, 2009).

Due to the successes of SIT in the control of lepidopteran species, and similarities in the ecology and biology between *T. leucotreta* (which is successfully controlled by an operational SIT program in South Africa) and *E. saccharina*, it is envisioned that a targeted SIT programme against *E. saccharina* could significantly reduce its population in South African sugarcane (Woods *et al.*, 2019[a]). Successful integration of SIT in the *E. saccharina* IPM program at SASRI, largely depends on rearing the pest on an artificial diet to provide a constant and large supply of partially sterile male moths, in sufficient numbers needed for effective SIT.

1.2 Mass rearing insects on artificial diets

Mass rearing of insects has expanded dramatically in the agricultural industry for development and support of IPM research (Leppla *et al.*, 2009). From as early as the 1980s various entomological literature emanating from SASRI has been published on rearing insects on artificial diet for research into host plant resistance, push-pull technology, biological control and SIT programmes (Conlong, 1992; Conlong, 1994; Conlong & Rutherford, 2009; Cockburn, 2013; Walton and Conlong, 2016; Ngomane *et al.*, 2017). In addition, books have been written outlining specific diets for different insect species (e.g. Singh, 1977), on how to develop insect diets (e.g. Cohen, 1992) and outlining the principles and procedures for rearing high quality insects (e.g. Schneider, 2009).

Rearing the pest on its natural host plant has many problems, which include the host plants seasonal availability, overall excessive costs to grow them in large enough quantities needed for mass production of insects, and variable insect quality of individuals feeding on the plants (Alfazairy *et al.*, 2012). The advantages of rearing insects under laboratory conditions on controlled scientifically derived diets are that colonies are uniform, offer high quality and disease-free

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organisms with a known rearing history and display consistent performance, assuming good nutrition and effective management of diseases (Roe *et al.*, 2017).

1.2.1 Artificial diets developed for plant feeding Lepidoptera species

The first known plant-feeding insect to be reared from egg to adult on artificial diet was the European corn borer, *Ostrinia nubilalis* Hubner (Lepidoptera: Crambidae) (Beck *et al.*, 1949). This diet subsequently formed the basis of many phytophagous insect diets (Davis, 2007). Hervet *et al.* (2016) further described the versatility of the McMorran diet, which was primarily used to rear species of Noctuidae. Adkisson *et al.* (1960) were the first to use wheat germ as an ingredient in artificial diet to rear *P. gossypiella*. The recipe was later modified by Vanderzant *et al.* (1962) to rear the corn earworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae). Berger (1963) further modified this diet to rear several noctuid insect species. McMorran (1965) modified this latter recipe to rear species of the tortricid family and Grisdale (1973) consequently added linseed oil to the recipe as an ingredient to reduce wing deformities in some lepidopteran species. Based on this recipe, Atkinson (1978) successfully developed the first artificial diet for *E. saccharina* in South Africa.

1.2.1.1 Artificial diets developed for Eldana saccharina

Diet development for mass rearing is a continuous process in IPM research, as demonstrated in the program against *E. saccharina* at SASRI. Following the work of Atkinson (1978), Graham and Conlong (1988), Rutherford and Van Staden (1991), Gillespie (1993), Walton and Conlong (2016) and Ngomane *et al.* (2017) all produced new diets. These diets continuously improved quality and production of insects needed for various IPM programs, whilst reducing production costs (e.g. Ngomane *et al.*, 2017). Table 1.1 outlines the different diets developed, and their formulations. The Ngomane *et al.* (2017) diet is currently used in the routine *E. saccharina* mass rearing program at SASRI.

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Table 1.1 Ingredients and amounts previously used in artificial diets for mass rearing *Eldana saccharina* larvae at the South African Sugarcane Research Institute.

Ingredients	Unit	Atkinson (1978)	Graham & Conlong (1988)	Rutherford & Van Staden (1991)	Gillespie (1993)	Walton & Conlong (2016)	Ngomane *et al.* (2017)
Filler							
Dried crushed sugarcane	g		7.33				
Proteins & carbohydrates							
Rabbit meal	g						66.67
Wheat bran	g						11.11
Ground chickpea	g	120.00	40.00		31.36	31.91	16.67
Casein	g	12.00	4.00		5.46	5.47	
Full cream milk powder	g						7.33
Whole egg powder	g						8.89
Glucose	g	20.00	6.67				
Sucrose	g						17.78
Undamaged/damaged sugarcane	DM%			30.00/25.00			
Vitamin & minerals							
Yeast extract	g				0.96	0.96	1.11
Brewers yeast	g	12.00					
Calcium lactate	g				0.36	0.36	0.36
Ferric citrate	g				0.02		
Sodium chloride	g				0.18	0.18	0.18
Vitamin mixture	g			0.66			
Lipids							
Oil/fatty acid mixture	g			1.18			
Preservatives & Antimicrobial agents							
Ascorbic acid	g	4	1.33		1.06	1.07	1.78
Sorbic acid	g	2	0.67	0.23			
Chloromycetin (Chloramphenicol)	g	0.7					
Nipagin (Methylparaben)	g	1.6	0.53	0.9	0.64		1.78
Sodium propionate	g				2.91	2.92	2.84
Formalin (40%)	ml		0.4	30	1.12	0.64	
Acetic acid	ml						2.22
Benomyl	g		0.01	0.06			
Dithane M45	g				0.05	0.06	0.06
Terralon LA	ml						0.4
Streptomycin	g			0.07			
pH modifiers							
Tri-sodium citrate	g				0.73	0.73	0.71
Citric acid	g				0.73	0.73	0.71
Gelling agents							
Agar powder	g	20	3.33		1.27	1.6	1.42
Fibrous cellulose	g	28					
Solvents/moisture							
Ethanol	ml			120	11.15		
Methanol	ml		16.67				
Denol (70%)	ml					11.17	10.89
Total		**220.30**	**80.94**	**161.20**	**122.23**	**121.63**	**152.73**
Water for agar	ml				98.00	213.00	133.00
Water balance	ml	220.30	333.00	200.00	221.00	106.00	178.00
Total diet volume	**ml**	**440.30**	**413.94**	**361.20**	**441.23**	**440.63**	**463.73**

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1.3 Functional aspects of insect artificial diet components

In order to develop artificial diets that are effective, a knowledge of the functional aspects of the diet nutrient components needed by the insects is essential. In addition, the balance of nutrients such as carbohydrates, proteins, lipids, vitamins and minerals in artificial diets developed for insects, is important because it directly influences insect growth, tissue maintenance, reproduction and energy allocation (Genc, 2006). Water is also a fundamental component. The concepts of water content and water activity (a_w) are essential because they help clarify how artificial diets work, why they sometimes fail and are responsible for contamination regulation (Cohen, 2015). Other commonly added ingredients important in determining diet quality include emulsifiers and gelling agents, stabilisers, pH modifiers, preservatives and antimicrobial agents (Schneider, 2009; Cohen, 2015) as they influence diet toughness and texture, which may compromise the diet's nutritional value, palatability and thus consumption of the diet by the insect (Karowe and Martin, 1993; Schneider, 2009). The diet ingredients given in Table 1.1 are subdivided into these components as examples.

1.3.1 Carbohydrates

Carbohydrates serve as building materials, energy sources and often act as feeding stimulants in insect diets. They are essential for optimum growth and development, reproductive activity and survival of the insects. Also, the primary structure of an insect's body consists mainly of a polysaccharide (chitin) made of amino sugars (Genc, 2006; Schneider, 2009; Cohen, 2015). Certain phytophagous insects belonging to the genera of *Ephestia* (Lepidoptera, Pyralidae), *Oryzaephilus* (Coleoptera, Silvanidae) and *Tenebrio* (Coleoptera, Tenebrionidae) fail to thrive on diets that have less than 50 % carbohydrates. For most insect species, glucose, fructose and sucrose are nutritionally adequate carbohydrate sources (Rockstein, 1978; Cohen, 2015). Some carbohydrates such as cellulose cannot be digested by insects but may be useful as a bulking ingredient and help promote intestinal mobility (Cohen, 2015).

1.3.2 Proteins and amino acids

Insects require optimum levels of proteins for best growth. Most insects digest proteins (polypeptides) from their food, which get broken down into amino acid components and absorbed and distributed to cells where they are resynthesized into proteins that also make up the insect's body (Cohen, 2015). Insects use proteins as their principle source of nitrogen, for structural purposes, and as enzymes for transport and storage (Genc, 2006; Cohen, 2015). Most adult female insects need protein to mature their ovaries and eggs, and males of many insect species require proteins for adequate longevity (Offor, 2010). As a rule, insects require a dietary source of 8 to 10 essential amino acids (methionine, threonine, tryptophan, valine, isoleucine, leucine, phenylalanine, lysine, arginine and histidine) and in the absence of one of these, growth and development may be inhibited (Genc, 2006; Cohen, 2015). Some amino acids are important in morphogenesis, others are known to be neurotransmitters and proline for example is essential for development and serves as an energy source to support flight (Genc, 2006).

1.3.3 Lipids

Lipids in biological organisms consists of fatty acids, alcohols, sterols and phospholipids (Genc, 2006). These are good sources of energy, in addition to functioning as building blocks of cell membranes, hormones, nutrient transporters and structural materials (Cohen, 2015). Studies have shown that more than 50 insect species, including lepidopterans, require a dietary source of polyunsaturated fatty acids (e.g. palmitic, oleic, linoleic oils etc.) and deficiencies in these result in wing deformities as their scales adhere to pupal cases on emergence (Genc, 2006; Schneider, 2009). Furthermore, phospholipids have been proven to increase fecundity when incorporated into artificial diets developed for phytophagous insects, and carotenoids have an effect on the coloration of certain insects (Offor, 2010; Cohen, 2015).

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1.3.4 Vitamins and minerals

Vitamins and minerals play an important role in insect diets and although our understanding of their requirements is limited, insects need trace amounts of these nutrients for their functioning (Cohen, 2015). Insects cannot synthesize vitamins but require a good source of thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, folic acid and biotin (obtained directly from their host plants) as cofactors to help enzymes catalyse metabolic pathways (Genc, 2006). Nutritional deficiencies of these vitamins commonly result in poor growth rates, lowered fecundity or fertility and reduced body weight (Genc, 2006; Cohen, 2015). Minerals in insect diets are intentionally added as salt mixtures (e.g. Beck's salt) (Cohen, 2015). Sodium, potassium, calcium, magnesium, chloride and phosphate are essential minerals for insects and function as co-enzymes in purine metabolism (Genc, 2006). The balance of these minerals was found to support the development of most corn borers and other lepidopteran insects (Offor, 2010).

1.3.5 Emulsifiers and gelling agents

Emulsifiers and gelling agents act as stabilisers, allowing lipid-phase materials and aqueous-phase materials to mix. They help preserve the mixed state of the ingredients and prevent reactions taking place between ingredients (Cohen, 2015). This helps accommodate solid substrate-feeding insects and prevents food from collapsing on insects as they feed inside the diet (Cohen, 2015). There are two classes of emulsifiers in insect diets: natural ones which include proteins (e.g. egg yolk, milk and soy proteins) and phospholipids (e.g. soy lecithin) and artificial ones which include polyoxyethylenesorbitans (Cohen, 2015). Some gelling agents such as proteins, starches and pectin can be used as nutrients, while others such as agar and carrageenan gel are non-digestible but contribute as texturizing agents (Cohen, 2015).

1.3.6 Antimicrobial agents

The success of mass rearing insects on artificial diet is limited by microbial (i.e. bacterial, yeast, mould, fungal, viral etc.) contamination. Microbial contamination alters the nutritional value of a diet, resulting in multiple effects on insect quality. These include reduction in insect health and size, prolonged development, increased mortality and reduced production of essential fatty and amino acids (Sikorowski and Lawrence, 1994; Sridhar and Sharma, 2013; Nair *et al.*, 2019). Most often protective ingredients are added to insect diets to prevent microbial contamination, oxidation or other means of nutrient destruction. These include antibacterial agents (e.g. streptomycin sulphate and chlortetracycline), antifungal agents (e.g. sorbic acid, methyl paraben, propionic acid and formalin) and antioxidants (e.g. ascorbic acid, tocopherol and butylated hydroxytoluene) (Cohen, 2015).

1.3.7 Importance of the diet's pH

The pH imparts several features to insect diets. It influences diet palatability, stability in relation to microbial contaminants, activity of preservatives, solubility of nutrients and functioning of enzymes (Karowe and Martin, 1993; Cohen, 2015). In general, insects prefer a slightly acidic pH range in the diet and they have the ability to regulate pH to support the acidic environment they encounter in the food and the intestinal pH changes caused by the type of food they ingest (Cohen, 2015; Dias *et al.*, 2019). Most antifungal agents only work at an acidic pH and bacterial growth on insect diets is also known to be suppressed at low pH (Cohen, 2015). Substances commonly used in insect diets to lower pH include hydrochloric acid, acetic acid, phosphoric acid, benzoic acid, citric acid, lactic acid, formic acid and tartaric acid. Bases used to raise diet pH include sodium hydroxide, potassium hydroxide, sodium carbonate and sodium bicarbonate (Cohen, 2015).

1.3.8 Water content (%) and water activity (a_w)

Most organisms need water contained in their food, or from a drinking source to sustain life processes. In insect rearing programs, unintentional creation of water stress can be disastrous and

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lead to shortcomings in insect rearing (Cohen, 2015). Insect artificial diets should contain the normal amount of water present in the insect's natural diet. For example, certain species of leaf feeders (i.e. cabbage loopers or beet army worms) are adapted to food that is about 90 % water and at anything less than that, insects would be stressed (Cohen, 2015). High nitrogen content in the diet also increases water stress, even if the water percentage is right. Such a diet can cause an insect to get rid of excess nitrogen waste forcing it to excrete excessive amounts of water (Cohen, 2015). Conversely, providing too much water can result in nutritional stress for insects adapted to feeding on diets that are concentrated in nutrients (Cohen, 2015).

Water activity (a_w), plays an important role in the diet's stability, shelf life, handling characteristics, physical properties, susceptibility to microbial contamination and chemical stability (Cohen, 2015). Free water in a diet supports microbial growth, participates in chemical and enzymatic reactions and supports spoilage processes (Rockland and Nishi, 1980). Water activity values range from 0.00 to 1.00 (a_w of water is 1.00) and according to Rockland and Nishi (1980), the water activity level known to limit bacterial growth is 0.90 a_w, 0.70 a_w for spoilage moulds and the lowest limit for microorganisms such as yeast is 0.60 a_w.

1.4 Preparation methods of insect artificial diets

1.4.1 Diet preparation

There are several flexible ways of preparing artificial diets for insect rearing, however, very few of these are common practices. Dyck (2010) provides a general guideline of methods for preparing insect artificial diets. Heating plays an important role in destroying microbial contaminants, detoxifying soy proteins, activating starch formation and gelling reactions and hydrating fillers such as soy meal (Dyck, 2010). Heating water between 52 ˚C up to 100 ˚C and adding gelling agents such as agar to the boiling water is required. Vitamins such as ascorbic acid should be added at temperatures not more than 60 ˚C. Carbohydrates such as sucrose, wheat germ and flour need to be added in the diets at temperatures between 52 ˚C and 90 ˚C. Some ingredients such as ethyl-hydroxyparaben and sorbic acid should be dissolved in ethyl alcohol and ascorbic acid needs to be dissolved in water (Dyck, 2010). Other diets are covered with wax film to prevent dehydration (Stenekamp, 2011).

1.4.2 Storage of diet ingredients and finished diets

Storage temperature is one of the most important factors affecting the stabilities of diet ingredients and completed diets. In general, storage at low temperatures (2-10 ˚C) is preferred to storage at high temperatures (Cohen, 2015). At high temperatures, changes in stored food such as syneresis (expulsion of liquid from one compartment to another), microbial degradation, oxidation, enzymatic and non-enzymatic chemical reactions and desiccation takes place, and at low temperatures microbial growth and the rate of degrading chemical reactions is reduced (Cohen, 2015). Light is also destructive to most diet ingredients and therefore, desirable storage conditions are cold, dry and dark places with low oxygen levels (Cohen, 2015). High water activity (a_w) influences the reactivity to oxygen and contributes to the destruction of nutrients in completed diets (fully hydrated diets) (Rockland and Nishi, 1980; Cohen, 2015). Thus, to further stabilise storage, it is important that water activity is lowered (Cohen, 2015). Changes in the diet's appearance, aromas, mouth feel and taste displays changes in the nutritional quality of stored food, and insects feeding on these diets have a natural sense of what is potentially harmful to them (Cohen, 2015).

For most dry ingredients (a_w < 0.50, moisture content < 10 %), storage at < 0 ˚C should preserve their nutritional value and palatability for months (Cohen, 2015). However, storing complete diets or ingredients with high water content at temperatures below 0 ˚C, have consequences, some of which arise from the choice of storage equipment. These consequences include (1) sublimation (water evaporation from its frozen state), which could be avoided by storing frozen materials in tightly packaged, waterproof containers and also by reducing storage time of the diets and ingredients, and

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(2) freezing, which degrades diets and ingredients by separating water from solutes, causing changes in pH concentrations and access of enzymes to substrates (Cohen, 2015). Freezing also forms ice crystals, which disrupt the integrity of the naturally protective compartments that characterize diet components, thus affecting the shelf life of the diet (Cohen, 2015).

1.5 Quality control in mass insect rearing

Quality control has become an established component in insect rearing programmes, as it assures that the quality of insects produced or the process of insect production is maintained or improved (Schneider, 2009; Cohen, 2015). Quality control in insect rearing includes production, process and product control. By implementing quality control, significant gains in efficiency and effectiveness are expected. On the other hand, disregard for quality control could lead to substantial increases in the programmes cost and a lack of effective pest control.

1.5.1 Production and process control

Production control addresses the inputs to rearing. These include performing and monitoring standard operational procedures developed for specific insects, and selecting and training employees. It is important that rearing personnel have ample understanding of the biology and behaviour of insects and associated pests and pathogens, and how these affect product quality. Further production control is needed to maintain rearing facilities and equipment. Sanitation and monitoring for microbial contamination are also important production control parameters. Rearing processes and quality control tests are the final production control parameters that need constant application. Failure to implement production control often declines insect production and quality (Schneider, 2009). Process control is often coupled with production control. It measures how things are done, including diet preparations, environmental conditions (i.e. temperature, humidity, photoperiod and air movement), irradiation doses etc. (Stenekamp, 2011). Quality control data can be processed and presented as tables, histograms, pie charts or graphs (Schneider, 2009).

1.5.2 Product quality control

Product quality control refers to the tests conducted to assure that insect production meets acceptable quality specifications and standards before the insects leave the production facility (Schneider, 2009). Routine insect quality parameters usually undertaken to assess the quality of the diet include, egg or larval mortality, survival, pupal weight, adult emergence, sex ratio, fecundity, fertility, longevity, thermal tolerance and insect flight performance (Stenekamp, 2011; Chidawanyika and Terblanche, 2011). The chemical and physical properties of artificial diets such as nutritive elements, contaminants, moisture, texture and pH can influence insect quality parameters (Karowe and Martin, 1993; Schneider, 2009; Stenekamp, 2011). Undernourishment and temperature affect the development time of larvae and reduces insect body weight. Temperature, humidity and the age of females influence fecundity and egg mortality (Chidawanyika and Terblanche, 2011). The percentage of adult emergence determines the number of insects to be released. Adult diet (e.g. water), temperature and humidity influence thermal tolerance, insect flight performance and longevity (Chidawanyika and Terblanche, 2011; Stenekamp, 2011).

1.6 The Carcass Milling Technique for effective diet formulation and rearing of *Eldana saccharina*

Most insect artificial diets are composed of high energy producing nutrients (De Goey, 1973; Sahtout, 2012). Insects consume approximately 70 to 75 % of the diet to provide energy for their life stage maintenance, and since diet expense is relatively higher than insect production costs, it is essential to avoid over-supply of nutrients, because once the insect's nutrient requirements are supplied, any excess nutrients are excreted or stored as unwanted fat by the insect, and are thus wasted (De Goey, 1973; Sahtout, 2012). Oversupply of nutrients also contributes to the build-up of primary or

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secondary metabolites which may be toxic, antagonistic or result in imbalances that lead to increased metabolic stress in the insect. Furthermore, undersupply or the absence of nutrients for the reared insects may lead to a total break in production, whereas minimal supply results in immune suppression, reduced productivity or a reduction in fecundity and fertility (Woods *et al.*, 2019[b]). However, to minimise these shortfalls in diet production, several techniques have been developed to evaluate actual nutritional requirements of the animal or insect to be fed, to help better formulate insect diets (Woods *et al.*, 2019[b]).

The use of a version of the comparative slaughter technique, a method for determining energy retention in animals, better described in the context of this project as the carcass milling technique, plays an important role in the development of animal feeds and most recently, insect diets (Woods *et al.*, 2019[b]). This technique requires that representative animals or insects (and their natural food) are slaughtered and analysed for dry matter, crude protein, crude fat, and energy using proximate and amino acid analyses (Babinszky and Barsony, 2013; Woods *et al.*, 2019[b]). The technique is expensive when applied to large animals but cheaper when applied to insects. It has been successfully used to determine baseline nutrient specifications for mass-rearing *T. leucotreta* and the black soldier fly, *Hermetia illucens* Linnaeus (Diptera: Stratiomyidae) (Woods *et al.*, 2019[a] and [c]). Using information collected from the proximate and amino acid analyses, relatively inexpensive artificial diets for insects have been formulated using feed formulation programmes such as WinFeed (Windows-based feed formulation program developed by EFG Software) (Woods *et al.*, 2019[b]).

Several artificial diets have been developed for mass-rearing *E. saccharina*. The diets developed effectively supported optimum survival and development of *E. saccharina* for the purposes they were developed. For example, Atkinson (1978) developed the first diet to provide material for biological studies of the insect. As the biological control program against *E. saccharina* gained momentum, higher numbers of this pest had to be reared to provide hosts for the respective biocontrol agents and material for plant resistance trials (Graham and Conlong, 1988; Gillespie, 1993). A complete artificial diet for bioassay purposes was developed (Rutherford and Van Staden, 1991; Rutherford *et al.*, 1994) and more recently, the diet was again improved for Sterile Insect Technique trials (Walton and Conlong, 2016; Ngomane *et al.*, 2017).

The diet developed by Ngomane *et al.* (2017) was based on a diet developed for *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) (Nagy, 1970). This diet was formulated based on ingredient composition and not formulated on the nutrient requirements of growing *E. saccharina* (Woods *et al.*, 2019[b]). The current study, therefore, provides a different approach to developing *E. saccharina* diets, based on animal science principles using the carcass milling technique, and body and chemical composition studies. The aim of this technique in diet production is to formulate the chemical composition of these diets unique to the particular insect being reared, to provide the optimum balance between carbohydrates, proteins, lipids, vitamins and minerals, in addition to specific requirements for sterols and essential amino acids needed by that specific insect.

1.7 Impact of sterols on the development and reproduction of *Eldana saccharina* reared on a carcass milling derived artificial diet

Rearing *E. saccharina* on an artificial diet and under laboratory conditions rather than on their natural host plants has proven beneficial for research purposes and pest control programs in the past (Conlong, 1992). While rearing the pest under controlled conditions, it is important that the insects produced display characteristics similar in viability, vigour and behaviour to wild populations, and they should demonstrate corresponding resistance to pathogenic organisms (Babu *et al.*, 2018). The loss of vigour and viability of laboratory reared populations is a well-known problem, often resulting in the collapse of insect colonies (Babu *et al.,* 2018). In many cases, after 4 to 5 generations, insect colonies experience reduced survival, growth rate, fecundity, fertility and hatching success. They

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also experience difficulties in moulting under certain environmental conditions (Babu *et al.,* 2018). The following are possible factors that could mitigate this.

Sterols play an important role in the physiological process of insects and greatly influence their growth and development (Behmer and Nes, 2003; Babu *et al.*, 2018). They are essential components of plant and animal cell membranes and serve as hormone precursors and signalling molecules for phytophagous insects (Nagata *et al.*, 2006; Behmer *et al.*, 2011; Babu *et al.*, 2018). The profiles of sterols differ among plants and animals. The most dominant sterol in animals is cholesterol. In plants small amounts of cholesterol are present but their dominant sterols are phytosterols (e.g. sitosterol, stigmasterol) (Janson *et al.*, 2009; Bouvaine *et al.*, 2014). Unlike most animals, insects are unable to synthesize sterols *de novo*, and depend directly on a dietary supply of these nutrients (Jing *et al.*, 2013; Babu *et al.*, 2018). The capability of phytophagous insects to utilize phytosterols differs significantly among insect species, for example, certain plant hoppers (Hemiptera) and beetles (Coleoptera) derive sterols from fungal endosymbionts, whereas herbivorous insects such as caterpillars (Lepidoptera) and grasshoppers (Orthoptera) generate tissue cholesterol by metabolizing phytosterols found in plant diets (Behmer *et al.*, 2011).

Cholesterol incorporated into the diet usually satisfies the sterol requirements for most insect species, even in trace amounts (Behmer and Nes, 2003; Babu *et al.*, 2018). Studies have shown that diets containing the phytosterols, sitosterol and stigmasterol, increased larval weight, growth rate and decreased mortality in lepidopteran species including the silkworm, *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae) and the south-western corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae). They have also reported that these sterols are essential as nutrients and feeding stimulants for insects (Al-Izzi and Hopkins, 1982). A deficiency of sterols in artificial diets greatly contributes to reduced egg hatch, inability of insects to moult and increased mortality in early instars (Babu *et al.*, 2018).

The current study further investigated whether sterols added to the carcass milling derived artificial diet had an added beneficial effect on the growth, development and reproduction of *E. saccharina*, with the aim to further improving its mass-rearing at SASRI.

1.8 Enhancing cold hardiness of *Eldana saccharina* moths through the addition of cryoprotectants to a carcass milling derived artificial diet

When mass-rearing insects under controlled laboratory conditions, challenges in insect field performance may arise, especially when rearing is intended for field releases in SIT programmes. These challenges occur due to laboratory adaptation, especially through reduced biological fitness caused by inbreeding, unintended selection or through direct rearing effects such as crowding and artificial diet (Sorensen *et al.,* 2012; Hoffmann and Ross, 2018). However this is remedied by re-introduction of individuals from the wild (a common practice in mass rearing) despite the production bottlenecks they may result, it is a necessary step (Mudavanhu *et al.*, 2016; Walton and Conlong, 2016).

In insect rearing facilities, selection pressure is mostly directed towards parameters which are important for culture productivity such as high fecundity, high fertility, short life cycle, large size etc., which is not necessarily a guarantee for optimal field performance. A high quality, reproductive insect reared in a mass-rearing facility could easily be a poor performer in the field, especially since reproductive output and field performance such as survival under stressful environmental conditions, flight ability and mating competitiveness might be traded off. So, for most control methods in IPM research, some form of quality control or ‘filtering’ for specific trait(s) is required to aid and/or enhance effective field performance or other required fitness traits of laboratory reared insects (Chidawanyika and Terblanche, 2011; Sorensen *et al.*, 2012).

Temperature plays an important role in the development, activity, distribution, abundance and survival of insects in the field. Insects are generally vulnerable to temperature variation mostly

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due to their small size and ectothermic physiology (Chidawanyika and Terblanche, 2011; Terblanche *et al.*, 2017). Understanding variation in thermal tolerance of insect pests is important for IPM programs. The physiological tolerance of insect populations to temperature are often associated with local climate. One crucial characteristic of climate identified to potentially limit species distribution is low-temperature performance or tolerance (Kleynhans *et al.*, 2014). In tropical areas, insects exposed to 10-15 °C may result in a chill-coma (cold-induced paralysis) or death, whereas insects in temperate and polar-regions may remain active and are able to fly at much lower, even sub-zero temperatures. Such insects compensate for potentially stressful temperatures by continuously adjusting their physiology and behaviour in order to survive and optimize their individual fitness in the environment (Lee and Denlinger, 1991). Depending on insect sensitivity, small changes in temperature may result in large differences in an insect's metabolic rate or respiratory water loss, growth rate and phenology (i.e. timing of seasonal activities) (Kleynhans *et al.*, 2014).

According to Kostal *et al.* (2011), physiological mechanisms fundamental to the ability of *Chymomyza costata* (Zetterstedt) (Diptera: Drosophilidae) larvae to survive freezing and cryopreservation at -196 °C remains poorly understood. However, the process of cold acclimation and freezing tolerance of insects generally involves significant biochemical changes, such as rapid increases in concentrations of cryoprotectants (e.g. proline and trehalose) and increases in relative proportions of phospholipids that couple palmitic and linoleic fatty acids in cell membranes. The rapid increases in these likely contribute to the preservation of proteins and membrane structures and insect function at low temperatures (Kostal *et al.*, 2011).

At SASRI, *E. saccharina* is routinely reared under laboratory conditions to support research and pest control programs (Conlong, 1992). Artificial diets for mass rearing the pest have been developed (Table 1.1) and these diets effectively supported optimum survival and development of *E. saccharina* for the purposes they were developed. Growth parameters and fitness traits of the pest have also been investigated by the authors on their developed diets. However, little is known on how the current and past laboratory diets, or host plants, influenced low temperature tolerance of *E. saccharina*. This understanding is important, as it influences field and low temperature activity, and also mass rearing and subsequent field performance. It could even explain the importance of being cold temperature tolerant while living in a sub-tropical climate.

To further this knowledge, the current study investigated whether cold hardiness of *E. saccharina* moths could be enhanced through the addition of cryoprotectants (i.e. amino acid and carbohydrate) into the carcass milling derived artificial diet. Thus, moths from these diets were exposed to short periods of cold stress, and recovery time after transfer to room temperature was recorded to determine which diet supplement might improve cold hardiness of individuals without having negative adverse effects on key quality parameters such as mating frequency, fecundity and fertility.

1.9 Aims and objectives of the study

The overall aim of the study was to further improve the routine diet currently used to rear *E. saccharina* at SASRI (Ngomane *et al.*, 2017), in terms of nutrition and insect production for mass propagation of *E. saccharina* adults fit for the SIT programme.

To attain the above aim, specific objectives of the study were as follows:

i. To formulate a new artificial diet based on the carcass milling technique and then compare growth and fitness parameters of *E. saccharina* reared on this diet and that of the diet developed by Ngomane *et al.* (2017).

ii. To investigate if cholesterol (cholest-5-en-3β-ol) and stigmasterol (stigmasta-5,22e-dien-3β-ol) improved *E. saccharina's* growth, development and reproduction on the best diet determined by point i.

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iii. To address whether cold hardiness of *E. saccharina* moths could be enhanced though the addition of a cryoprotective amino acid, L-proline ((s)-pyrrolidine-2-carboxylic acid) and a carbohydrate, trehalose (α-d-Glucopyranosyl-α-d-glucopyranoside) into the best diet determined by point i.

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Chapter 2. Materials and Methods

2.1 Experimental site

The study was conducted at the SASRI-Insect Rearing Unit (IRU), Mount Edgecombe, in KwaZulu-Natal, South Africa. This rearing facility provided reliable temperature and humidity control, and light through windows supplemented by T12 65 W cool white fluorescent tubes (Philips, Philips Lighting, Poland). Laboratory standard operational procedures developed for the SASRI-IRU were followed to prevent contamination and contamination spread in the diets being tested (Ngomane *et al.*, 2017). All temperature and relative humidity conditions were kept similar for all aspects tested for both the formulated artificial and control diets (26 ± 2 °C, 72 ± 5 % RH). However, photophase was different for rearing (zero L: 24-hour D), quality (8-hour L: 16-hour D), chill coma recovery (conducted during the day) and oviposition assessments (8-hour L: 16-hour D). The 0 L: 24-hour D photophase was used as larvae were the life stage being subjected to the diets, and these are generally cryptically feeding inside stalks thus light could not reach them. On the other hand, the chill coma recovery and oviposition trials were done on adults, who were exposed to daylight in their natural life cycle, hence the 8-hour L: 16-hour D photophase.

2.2 Carcass Milling Technique

2.2.1 Collection and treatment of *Eldana saccharina* larvae

Eldana saccharina larvae routinely reared on the Ngomane *et al.* (2017) diet, in the SASRI-IRU larval growth room (maintained at 28 ± 2 °C, 72 ± 5 % RH and zero light: 24 hour dark photo phase), were harvested by hand from diet trays (inoculated 15 to 20 days before) and separated into different larval instar groups (i.e. 2nd instar, 3rd/4th instar, & 5th/6th instar). The collected 2nd instar larvae were exposed for 1 minute in boiling water (100 ºC), and the 3rd/4th and 5th/ 6th instar larvae were exposed for 1.5 minutes. These exposure times were determined so that the boiling water did not denature their larval proteins. Underexposure to the boiling water, in contrast, would allow the larval normal metabolic activity to continue, thus also giving an erroneous protein reading (Woods *et al.*, 2019[b]). After exposure, larvae were cooled rapidly and stored in a Bio Compact freezer (BioCompact II 410, Gram Commercial, Denmark) at -20 ˚C. A sample of 50 g of larvae per instar group was required for the proximate and amino acid analysis. This was packed separately into clean plastic vials (50 ml), lids screwed on tightly, sealed in a clear plastic sleeve (310 mm wide x 80 micron thick), labelled accordingly (i.e. larval instar group) and kept in a polystyrene cooler box (230 x 155 x 165 mm) packed with 3 frozen Seagull solid jumbo ice bricks (180 x 120 x 40 mm). The cooler box and contents were couriered overnight to the Department of Animal Sciences, Stellenbosch University for proximate and amino acid analyses to be completed.

2.2.2 Collection of natural host plants of *Eldana saccharina*

The bottom section (25 cm long) of 4 mature (12 months old) sugarcane stalks (variety NCo 376), cut into short pieces (to make up 200 g of fresh sample), were collected from SASRI field number 14 (GPS coordinates 29°42'15.25"S 31°02'40.93"E). Mature, fully expanded papyrus (*Cyperus papyrus* L.) umbel meristems (to make up 200 g of fresh sample) were collected on the SASRI property/waterways. The collected host plant materials were sealed separately in clear plastic sleeve bags just larger than the fresh plant material collected, labelled accordingly and stored in the Bio Compact freezer at -20 ˚C. The plant samples were kept separately in polystyrene cooler boxes (230 x 155 x 165 mm) each packed with 3 frozen Seagull solid jumbo ice bricks (180 x 120 x 40 mm). The cooler boxes and contents were couriered overnight to the Department of Animal Sciences, Stellenbosch University for proximate and amino acid analyses to be completed.

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2.2.3 Proximate and amino acid analyses

At the Department of Animal Sciences, thirty sub-samples of the larval cadavers and natural diets of the larvae (sugarcane and papyrus) were dried in an oven (Labcon FSOH 16, CC Imelmann, South Africa) at 60 °C for 24 hours. They were each homogenised using a mill (KN 195 Knifetec™, FOSS, Denmark) and subsamples were taken for their Ideal Amino Acid Profiles (IAAP) to be determined (Woods *et al.*, 2019[b]).

2.2.3.1 Amino acid analyses

The process involved weighing 0.1 g of sample, placing it in a specialised hydrolysis tube and adding 6 ml of hydrochloric acid (HCl) and 150 ml of phenol (Woods *et al.*, 2019[b]). The tubes were vacuated and nitrogen added under pressure. These were sealed off with a blue flame and samples left for 24 hours to hydrolyse at 110 °C (Woods *et al.*, 2019[b]). After hydrolysis, the samples were transferred to 0.5 ml Eppendorf microcentrifuge tubes and kept in the refrigerator (KIC 170 L Top Freezer/Fridge, KIC, South Africa) at 4 °C until they were ready to be sent to the Central Analytical Facility of Stellenbosch University, where amino acid composition was determined by means of the water AccQ Tag Ultra Derivation method (Woods *et al.*, 2019[b]).

2.2.3.2 Proximate analyses

Moisture, crude protein and ash of the natural diets and the different larval instar cadaver groups were analysed according to the methodology of the Association of Official Analytical Chemists (AOAC) (Woods *et al.*, 2019[b]). To determine moisture content, 2.5 g of the homogenised sample was weighed, dried in an oven (Labotec EcoTherm Digital Oven, Labotec, South Africa) for 24 hours at 100 – 105 °C and then re-weighed. The dried sample was incinerated in a muffle furnace (Thermo Scientific™ Muffle Furnaces, ThermoFisher Scientific, United States) for 6 hours incinerated at 500 °C. It was then cooled using a desiccator (Deschem Glass Vacuum Desiccator 8" (210 mm), Deschem Science Supply, United States) and re-weighed to provide an estimate of the ash content (Woods *et al.*, 2019[b]). To determine the total lipid content of the samples, a rapid solvent extraction method was used (Lee *et al.*, 1996). A ratio of 1:2 chloroform: methanol solution was used for the plant samples due to their relatively low fat content (< 50 g kg^{-1}, USDA, 2019) and a ratio of 2:1 chloroform: methanol solution was used for *E. saccharina* larval cadaver samples, since initial test determination of their lipid content was found to be above 50 g kg^{-1} (Woods *et al.*, 2019[b]). To determine the total protein content, the LECO combustion method was used (Woods *et al.*, 2019[b]). The dried defatted samples were crushed using a coffee grinder (Russell Hobbs – 120 W Multi-purpose Coffee Grinder, Russell Hobbs, United Kingdom), and for each sample an aliquot of 0.5 g was weighed into a LECO foil cup, which was incinerated and then analysed for nitrogen content using EDTA for calibration. To obtain the protein content, nitrogen content was multiplied by 6.25 (Woods *et al.*, 2019[b]).

2.2.4 Artificial diet formulation using WinFeed

Six artificial diets, namely minimum specification (MS), ideal amino acid profile of the 2nd instar larvae (IAAP2), ideal amino acid profile of the 3rd/4th instar larvae (IAAP3/4), ideal amino acid profile of the 5th/6th instar larvae (IAAP5/6), sugarcane (SC) and papyrus (PAP) diets, were formulated from the proximate and amino acid analyses using the WinFeed 3.0 (EFG, 2005) programme (Woods *et al.,* 2019[b]). The MS diet was formulated using the minimum amounts of common dietary ingredients obtained from a summary of published *E. saccharina* diets (Atkinson, 1978; Graham and Conlong, 1988; Rutherford and Van Staden, 1991; Gillespie, 1993; Walton and Conlong, 2016; and Ngomane *et al.*, 2017; Table 1.1), on which the insect was previously successfully reared (Woods *et al.*, 2019[b]). The IAAP2, IAAP3/4, IAAP5/6 diets were formulated using ideal amino acid compositions, determined from the carcass milling technique, which resembled the amino acid profiles of the different larval instar groups (Woods *et al.*, 2019[b]). The SC and PAP diets were

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formulated using the nutrient compositions, determined by proximate and amino acid analyses, of the natural diets of *E. saccharina*. The control diet was reverted to nutrient composition using WinFeed and the calculated nutrient composition was used as the control nutrient specifications (Woods *et al.*, 2019[b]). The experimental diets were formulated to unique nutrient specifications using similar ingredients to that of the control diet (Ngomane *et al.*, 2017).

To formulate for the MS diet, raw ingredients present in published *E. saccharina* diets (See Chapter 1, Table 1.1) were entered into the “ingredient” tab of the WinFeed programme. Cost (ZAR) per ton and bag weights (g) of the ingredients were also entered. All essential nutrients available in the ingredients were selected on the “nutrient” tab. The nutrient composition of each of the ingredients were obtained from various internet sources i.e. Feedipedia (https://www.feedipedia.org, viewed 4 June 2018), Food and Agriculture Organisation (FAO) (http://www.fao.org/home/en/, viewed 4 June 2018), google scholar (https://scholar.google.com/, viewed 4 June 2018) and google search (https://www.google.com/, viewed 4 June 2018). The amino acids and minerals entered into the programme were expressed as a percentage of protein sample and ash sample, respectively, on a dry matter basis. Dry matter percentage was entered As-Is. In the “animals” tab, the experimental insect (*E. saccharina*) was entered. All ingredients and nutrients were made applicable to the specified insect. Compositions of the diets found in literature were created in the “compositions tab”. For each diet composition, ingredients were selected, and their weights were entered. The diets were then brought to a scale of 100 %.

Once all the diet compositions were entered into WinFeed, a summary report on excel showing the compositions with their associated ingredients and ingredient prices, could be accessed. At the bottom of the table, the costs of the compositions were calculated. In the same excel file, a nutrients table showed all the available nutrients found in the diet compositions entered. Using this table, minimum specification values of the nutrients were calculated. Back on the WinFeed programme a feed was created. The minimum specification values were entered on the “feed” tab under “minimum”. Applicable nutrients and ingredients were selected. Once all the information was entered, the diet was formulated (Table 2.1). Another summary report could be assessed showing the newly formulated MS diet with associated ingredients, their nutrients and costs (Woods *et al.*, 2019[b]).

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2.2.4.1 Diet formulation for the carcass milling diets

Table 2.1 Ingredients and amounts required for preparing the formulated *Eldana saccharina* larval and control (ECBMOD) diets.

Formulated Diets vs Control Diet (ECBMOD)								
	Diet Name	**MS**	**IAAP2**	**IAAP3/4**	**IAAP5/6**	**SC**	**PAP**	**ECBMOD**
Ingredients	**Unit**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**
Carrageenan gel	g	15.00	15.00	15.00	15.00	15.00	15.00	
Agar powder	g							4.60
Lucerne meal	g	250.00	250.00	250.00	250.00	50.00	50.00	
Rabbit meal	g							226.40
Wheat bran	g	55.80	53.20	6.00	6.00		40.75	56.60
Yeast extract	g							3.40
Ground chickpea	g	53.00	54.60	85.20	85.20	84.75	81.30	56.60
Full cream milk powder	g	7.60	7.60	7.60	7.60	7.60	24.45	22.60
Whole egg powder	g	28.40	28.40	28.40	28.40	28.40	28.40	28.20
Sucrose	g	66.20	66.40	68.60	68.80	70.25	133.00	64.60
Sodium chloride	g					4.72	63.00	0.60
Nipagin	g	6.40	6.40	6.40	6.40	6.40	6.40	6.40
Sodium propionate	g	10.40	10.40	10.40	10.40	10.40	10.40	10.40
Oxytetracycline	g	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Ascorbic acid	g	6.40	6.40	6.40	6.40	6.40	6.40	6.40
Acetic acid	ml	8.00	8.00	8.60	8.00	8.60	8.60	8.00
Citric acid	g	2.60	2.60	2.60	2.60	2.60	2.60	2.60
Tri-sodium citrate	g	2.60	2.60	2.60	2.60	2.60	2.60	2.60
L-Lysine HCL	g						25.00	
Vit+min premix	g	0.80	0.80	0.80	0.80	0.80	0.80	
Total		**515.20**	**514.40**	**500.60**	**500.20**	**300.52**	**500.70**	**502.00**
Water for agar	ml							500.00
Water balance	ml	1500.00	1500.00	1500.00	1500.00	1500.00	1500.00	1000.00
Total diet volume	**ml**	**2015.20**	**2014.40**	**2000.60**	**2000.20**	**1800.52**	**2000.70**	**2002.00**

The formulated diets contained lucerne meal and the control diet contained rabbit meal (sourced from Mkondeni Animal Feeds CC, Pietermaritzburg, South Africa) as their main ingredients. These were crushed into powder, using a Baby Hippo Hammer Mill (SNU506-605, Collins and Son, South Africa) and sterilised in a force draft oven (Memmert UL80, Gemini BV, Netherlands) at 65 °C for 2 days. The control diet used agar as a gelling agent and the formulated diets used carrageenan gel as a cheaper alternative to agar (Woods *et al.*, 2019[b]). The control diet included yeast extract and the PAP diet included L-Lysine HCL, as an added protein source. The SC, PAP and control diets also include sodium chloride, which is critical for osmoregulation, neuromuscular mechanisms and digestion and excretion processes in insects (Xiao *et al.*, 2010). The remaining diet components commonly included carbohydrates and proteins (i.e. egg powder, chickpea flour, wheat bran, full cream milk powder and sucrose), vitamins and minerals (i.e. vitamin premix), pH modifiers (i.e. tri-sodium citrate and citric acid) and preservatives and antimicrobial agents (i.e. ascorbic acid, nipagin, sodium propionate, acetic acid and oxytetracycline).

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2.2.4.2 Formulation for sterol incorporation into diets

Table 2.2 The *Eldana saccharina* MS diet formulations with the incorporation of the different sterol components, and the ECBMOD control diet recipe.

MS Diets Containing Sterols vs Control Diet (ECBMOD)								
	Diet Name	**MS (0.1gC)**	**MS (1.0gC)**	**MS (0.1gS)**	**MS (1.0gS)**	**MS (0.2gC:0.2gS)**	**MS (0.5gC:0.5gS)**	**ECBMOD**
Ingredients	**Unit**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**
Carrageenan gel	g	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Agar powder	g							4.60
Lucerne meal	g	250.00	250.00	250.00	250.00	250.00	250.00	
Rabbit meal	g							226.40
Wheat bran	g	55.80	55.80	55.80	55.80	55.80	55.80	56.60
Yeast extract	g							3.40
Ground chickpea	g	53.00	53.00	53.00	53.00	53.00	53.00	56.60
Full cream milk powder	g	7.60	7.60	7.60	7.60	7.60	7.60	22.60
Whole egg powder	g	28.40	28.40	28.40	28.40	28.40	28.40	28.20
Sucrose	g	66.20	66.20	66.20	66.20	66.20	66.20	64.60
Sodium chloride	g							0.60
Nipagin	g	6.40	6.40	6.40	6.40	6.40	6.40	6.40
Sodium propionate	g	10.40	10.40	10.40	10.40	10.40	10.40	10.40
Oxytetracycline	g	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Ascorbic acid	g	6.40	6.40	6.40	6.40	6.40	6.40	6.40
Acetic acid	ml	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Citric acid	g	2.60	2.60	2.60	2.60	2.60	2.60	2.60
Tri-sodium citrate	g	2.60	2.60	2.60	2.60	2.60	2.60	2.60
Vit+min premix	g	0.80	0.80	0.80	0.80	0.80	0.80	
Cholesterol	g	0.10	1.00			0.20	0.50	
Stigmasterol	g			0.10	1.00	0.20	0.50	
Total		**515.30**	**516.20**	**515.30**	**515.60**	**515.60**	**16.20**	**502.00**
Water for agar	ml							500.00
Water balance	ml	1500.00	1500.00	1500.00	1500.00	1500.00	1500.00	1000.00
Total diet volume	**ml**	**2015.30**	**2016.20**	**2015.30**	**2016.20**	**2015.60**	**2016.20**	**2002.00**

Table 2.2 describes the minimum specification (MS) diet recipes containing different concentrations of cholesterol (C) and sigmasterol (S) (MS (0.1gC), MS (1.0gC), MS (0.1gS), MS (1.0gS), MS (0.2gC:0.2gS) and MS (0.5gC:0.5gS)). The cholesterol additive was cholest-5-en-3β-ol (cholesterol, ≥ 99 %) and the sigmasterol additive was stigmasta-5,22e-dien-3β-ol (stigmasterol, ≥ 98 %), purchased from Sigma-Aldrich Chemicals Company, Missouri, United States; and the modified *Ostrinia nubilalis* diet (ECBMOD) currently used to routinely rear *E. saccharina* larvae at the SASRI-IRU. The MS diets were formulated according to minimum nutrient specifications for mass-rearing *E. saccharina* determined by Woods *et al.* (2019[b]).

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2.2.4.3 Formulation for cryoprotectants into diets

Table 2.3 The *Eldana saccharina* MS diet formulations with the incorporation of the different cryoprotectant components, and the ECBMOD control diet recipe.

MS Diets Containing Cryoprotectants vs Control Diet (ECBMOD)								
	Diet Name	**MS (0.1gP)**	**MS (1.0gP)**	**MS (0.1gT)**	**MS (1.0gT)**	**MS (0.2gP:0.2gT)**	**MS (0.5gP:0.5gT)**	**ECBMOD**
Ingredients	**Unit**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**	**Wt.**
Carrageenan gel	g	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Agar powder	g							4.60
Lucerne meal	g	250.00	250.00	250.00	250.00	250.00	250.00	
Rabbit meal	g							226.40
Wheat bran	g	55.80	55.80	55.80	55.80	55.80	55.80	56.60
Yeast extract	g							3.40
Ground chickpea	g	53.00	53.00	53.00	53.00	53.00	53.00	56.60
Full cream milk powder	g	7.60	7.60	7.60	7.60	7.60	7.60	22.60
Whole egg powder	g	28.40	28.40	28.40	28.40	28.40	28.40	28.20
Sucrose	g	66.20	66.20	66.20	66.20	66.20	66.20	64.60
Sodium chloride	g							0.60
Nipagin	g	6.40	6.40	6.40	6.40	6.40	6.40	6.40
Sodium propionate	g	10.40	10.40	10.40	10.40	10.40	10.40	10.40
Oxytetracycline	g	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Ascorbic acid	g	6.40	6.40	6.40	6.40	6.40	6.40	6.40
Acetic acid	ml	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Citric acid	g	2.60	2.60	2.60	2.60	2.60	2.60	2.60
Tri-sodium citrate	g	2.60	2.60	2.60	2.60	2.60	2.60	2.60
Vit+min premix	g	0.80	0.80	0.80	0.80	0.80	0.80	
L-Proline	g	0.10	1.00			0.20	0.50	
Trehalose	**g**			**0.10**	**1.00**	**0.20**	**0.50**	
Total		515.30	516.20	515.30	515.60	515.60	16.20	502.00
Water for agar	ml							500.00
Water balance	ml	1500.00	1500.00	1500.00	1500.00	1500.00	1500.00	1000.00
Total diet volume	**ml**	**2015.30**	**2016.20**	**2015.30**	**2016.20**	**2015.60**	**2016.20**	**2002.00**

The minimum specification (MS) diet was used again as the base diet into which two different synthetic cryoprotectants, the amino acid proline (L-proline ((s)-pyrrolidine-2-carboxylic acid; P) and carbohydrate trehalose (α-d-Glucopyranosyl-α-d-glucopyranoside; T) (purchased from Sigma-Aldrich Chemicals Company, Missouri, United States), at different concentrations were added (e.g. MS (0.1gP), MS (1.0gP), MS (0.1gT), MS (1.0gT), MS (0.2gP:0.2gT), MS (0.5gP:0.5gT)). The modified *Ostrinia nubilalis* diet (ECBMOD) was used as the control diet. Their formulations are given in Table 2.3.

2.3 *Eldana saccharina* rearing process

2.3.1 Diet preparation

Diet ingredients were stored in a cold room at 3 °C before use. They were weighed, using a calibrated two decimal place balance (Mettler Toledo ML6001 New Classic MF), according to the formulated diet recipes (Table 2.1, 2.2 and 2.3) in the IRU diet kitchen, maintained at 22 ± 2 °C and ambient humidity. The weighed dry content ingredients of each formulated diet was poured into the bowl of a food mixer (6.7 L Kenwood Titanium Major KMM060) and thoroughly mixed for 1 minute. Boiling

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water at a ratio of 1:3 (500 g : 1500 ml dry matter to water), together with 8.00 ml of acetic acid (dissolved in water), was added to the mixture. The food mixer was allowed to run for another minute. The resulting mixture (2 L of diet) was poured into a 2.5 L plastic mixing bowl and placed in a 700 W microwave oven (AMW17 Manual Microwave Oven) for 2 minutes on high heat to cook. Extra care was taken to ensure that the diet did not overcook. As soon as the diet started bubbling within the 2 minutes, it was removed from the microwave, thoroughly mixed using a sterilised spoon (dipped in Denol (70 %) and then in distilled water) and then placed back in the microwave to continue cooking until the 2 minute cooking period was completed.

The preparation of the control diet followed the same procedures, with the exception that the agar solution was prepared separately. In a 1 L plastic jug, 500 ml of boiling water was dispensed into which 4.6 g of agar powder was poured slowly and stirred using a spoon to avoid the formation of agar lumps. The agar solution was poured into a 1 L Schott Pyrex bottle and autoclaved at 121 °C for one hour. Once autoclaved, the hot agar was poured into the running food mixer and a balance of 1000 ml of boiling water with 8.00 ml of acetic acid was added to the autoclaved agar solution, followed by the weighed dry mixture (502.00 g). The resulting mixture was poured into a 2.5 L plastic mixing bowl and placed in a 700 W microwave oven to cook for 2 minutes on high heat. As soon as the diet started bubbling, it was removed from the microwave, thoroughly mixed using a clean spoon and then placed back in the microwave to continue cooking until the 2 minute cooking period was completed.

2.3.2 Diet dispensing

Seven hundred 25 ml plastic screw top vials, for each formulation described in section 2.2.4.1, 2.2.4.2 and 2.2.4.3, were sterilised overnight in a 0.5 % sodium hypochlorite (NaOCl) solution. Upon removal, they were shaken to remove most of the NaOCl solution and placed on a running laminar flow bench to dry, and further surface sterilised with ultraviolet germicidal lights behind ultraviolet (UV) resistant welding curtains on the laminar flow bench. After being cooked, but before the diets could set, 10 ml of diet was dispensed into each vial using a perimatic pump (Jencons Scientific Perimatic GP II Peristaltic Pump Dispenser, Cambridge Scientific Products, United States). Each diet formulation having a replication of 100 vials. Fifteen vials prepared per diet formulation were used to determine development time to first pupation and the remaining 85 vials were for harvest at full pupation. The vials with their dispensed diet were left to cool for 1 hour on a running laminar flow bench. Once cool, the diet surface in each vial was scarified using a sterilised dissecting needle (dipped in Denol (70%) and then in distilled water), breaking the skin of the diet surface, which allowed the eclosed larvae to enter the diet more easily. Two grams of sago (mixed with 0.0004 g of Dithane M45 as a fungicide) was poured over the surface of the diet in each vial. The sago helped absorb excess moisture on the surface of the diet and also served as a refuge for neonate larvae before they entered the diet.

2.3.3 Inoculation of neonate larvae onto the diet

Eldana saccharina eggs (oviposited on sheets of paper towelling and sealed in plastic bags just bigger than the paper towelling) were collected from the SASRI-IRU adult emergence and oviposition room (maintained at 28 ± 2 °C, 72 ± 5 % RH and at an 8-hour L: 16-hour D photo phase). The eggs were kept in an incubator (Laboratory Incubator CLN 32, Pol-Eko Aparatura, Poland) for 7 days, at 24 °C, 72 % RH and zero L: 24-hour D photophase, for them to hatch into neonate larvae. Two neonate larvae were carefully placed on top of the sago in each vial, using a fine paintbrush (size 00) dipped in Denol and then distilled water. The vials were sealed with lids that provided proper ventilation through stainless steel fine mesh gauze to prevent neonates from escaping, labelled accordingly (i.e. diet formulation, inoculation date, quality assessment dates) and placed in plastic storage baskets. They were kept in a larval growth room (maintained at 26 ± 2 °C, 72 ± 5 % RH and zero L: 24-hour D photo phase). The plastic storage baskets were stacked on clean 5-tier metal racks. A maxim iButton DS1923 (configured using the Fairbridge Technologies ColdChain

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thermodynamics software) was placed in the larval growth room with the inoculated vials, hanging on a tag on a 5-tier metal rack, to monitor the temperature and humidity in the room at 15-minute intervals.

2.3.4 Physical properties of artificial diets

The physical properties (pH, moisture content and water activity) of the different diets listed in Table 2.1, were tested once a week from the period of inoculation up until harvest. The physical properties of the MS diets listed in Table 2.2 and 2.3 were similar to those of the MS diet listed in Table 2.1 and therefore were not reported on in this study. The physical properties of this diet thus reflecting those of the former 2 diets. Diet contamination was also checked by visual observation. The most common contaminant is a green fungus (*Aspergillus* spp.) known to compromise diet quality and lead to increased mortality (Graham, 1990).

2.3.4.1 Diet pH testing

The diets pH was tested using a 2-point calibrated flat probe pH meter (211 Microprocessor pH Meter, HANNA Instruments, United States). The pH meter was first calibrated by inserting the probe into a neutral buffer solution (pH at 20 °C ± 0.1 °C of 7.00 ± 0.02). Once calibrated, the probe was rinsed with distilled water and inserted 3 cm into the diet of each vial. The pH was then recorded.

2.3.4.2 Moisture content determination

To determine moisture content of the diets, a 50 g sample of each formulation was baked dry to constant mass at 65 °C for 5 days. It was weighed daily using a calibrated two decimal place balance (Mettler Toledo ML6001 New Classic MF, METTLER TOLEDO Solutions, Europe) until constant mass was obtained. The difference in initial and final mass was assumed to be moisture loss and expressed as percentage of initial mass. Moisture lost by the diets over a 4-week period of larval growth was determined. Five empty vials per formulation (to be subtracted from the weights of the vials containing diet, to give the weight of the diet sample only) were weighed, 10 ml of diet from each formulation was dispensed into each vial and 2 g of sago was poured over the surface of the diets. The vials containing diet were then weighed to obtain initial weights. The diets were kept at 26 ± 2 °C, 72 ± 5 % RH and zero L: 24-hour D photo phase in a larval growth room. On a weekly basis the vials containing diet were re-weighed and recorded. Each week the weights obtained were subtracted from the previous weeks weights to determine mean moisture lost and the mean moisture lost over the 4-week testing period was subtracted from the initial moisture content (determined using the dry oven method).

2.3.4.3 Water activity measurement

The water activity in the diets was measured using a Decagon Pawkit water activity meter (Decagon Pawkit, Lab Cell, Ireland). In disposable 8 ml AQUALAB sample cups, four ml of diet per formulation were filled in each cup and the bottom of each cup was completely covered with the diet. Each prepared sample cup containing diet was placed onto a level surface. To take a measurement, the sensor cover of the Pawkit meter was flipped back and the Pawkit meter was placed onto the sample cup. The cup fitted over the sensors into a recess in the bottom of the Pawkit meter, making a vapour seal with the sensor. Once the Pawkit meter was properly positioned over the sample cup, readings of water activity within the diet samples were taken.

2.4 Quality assessment

Quality assessment was conducted on *E. saccharina* life stages, reared on the different diet formulations listed in Table 2.1, Table 2.2 and Table 3.3. The main objective for the diets developed in Table 2.2 was to investigate whether the addition of sterols into the carcass milling derived diet improved *E. saccharina's* growth, development and reproduction. Thus, only survival and population

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age distribution at the time to first pupation and at harvest, male and female pupal weight, moth emergence and sex ratio and female fecundity and fertility was determined. The main objective for the diets developed in Table 2.3 was to investigate whether cryoprotectants added into the carcass milling derived diet enhanced cold hardiness of *E. saccharina* moths. Thus, only male and female pupal weight, moth emergence and sex ratio and chill coma recovery time was determined. In addition male and female mating frequency, and fecundity and fertility of those adults chill coma imposed and not imposed, were compared.

2.4.1 Development time to first pupation

After 20 days in the larval growth room, life stages from the allocated 15 vials were manually and gently extracted from the diets in the vials using dissecting forceps, and divided into size categories (1st/2nd instar, 3rd/4th instar, 5th/6th instar, pre-pupal stage and pupal stage). These were placed into one of five 250 ml plastic jars with lids, according to its respective label. Each of the test diets, were assessed in this way. The respective life stages were counted using a tally counter after the diet assessment was completed. Dead insects from each diet were counted as they were found. Once all life stages were counted, the results were recorded.

2.4.2 Pupal harvesting and pupal weighing

To determine optimum pupal production, the remaining vials (85 vials per treatment) were harvested 27 days after inoculation. Ngomane *et al.* (2017) determined this to be peak pupal production time under similar laboratory conditions for their diet. Details of the harvested vials were recorded (i.e. diet formulation, date of inoculation, date of harvest and number of vials harvested) and the insect life stages (i.e. larvae, pre-pupa, pupae, moths and dead insects) collected were counted and recorded. Harvested pupae were placed singly into cells of multicell trays (n = 32 cells per tray) covered with ventilated plastic cling wrap film to prevent adult escape. Pupae were placed singly per cell to ensure that virgin males and virgin females were available for further experiments (i.e. Oviposition testing; Section 2.4.5). Trays were labelled with diet formulation and harvested date, stacked on multicell tray metal racks and stored in the IRU adult emergence and oviposition room (maintained at 26 ± 2 °C, 72 ± 5 % RH and 8-hour L: 16-hour D photo phase) on 5-tier metal racks. The trays were stacked on 5-tier metal racks to allow for sufficient ventilation and even distribution of temperature and humidity conditions.

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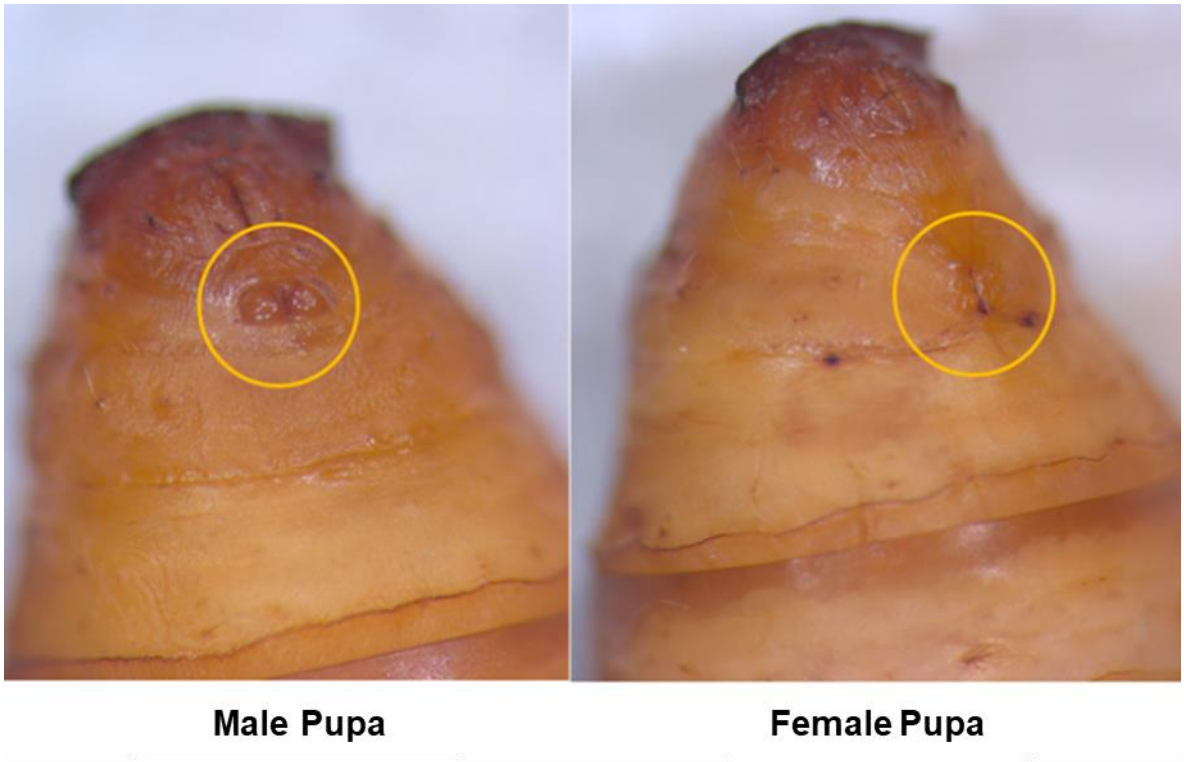

Figure 2.1 Male and Female *Eldana saccharina* pupae identified based on the different structures of their external genitalia (circled). The male pupae have two ball structures and female pupae have a small vertical line/slit on the ventral surface of their last abdominal segment.

Naked pupae (i.e. pupae carefully cut out of their cocoons) were collected from harvested batches of pupae for each diet formulation. Using a light dissecting microscope, 30 male and 30 female pupae per treatment were identified, based on the different structures of their external genitalia (Atkinson, 1980; Figure 2.1). The pupae of both sexes were separately weighed in grams using a calibrated four decimal place balance (Mettler Toledo ML54 Analytical Balance).

2.4.3 Adult emergence and sex ratio

Adult emergence and sex ratio counts were conducted from the pupae produced from the different diets. A day after harvest and on a daily basis thereafter (process took a maximum of 2 weeks), freshly emerged adult males and females were counted and recorded. Markings on the cells (M for males and F for females) were made to identify and separate insects that had emerged. From the emergence, the ratio of males-to-females in each diet formulation was determined.

2.4.4 Chill coma recovery assessment

Chill coma recovery was conducted to determine cold hardiness on *E. saccharina* moths reared on the different diet formulations. Freshly emerged male and female moths from each diet formulation were placed individually into air and watertight plastic vials (35ml). A total of 30 males and 30 females per diet formulation were used. Each individual exposed to chill coma was regarded as a repetition. The vials were sealed with lids and labelled accordingly (i.e. diet formulation and moth sex). The vials with moths were submerged in an ice-water slurry in a polystyrene cooler box (230 x 155 x 165 mm) for 2 hours. Temperature of the ice-water slurry (i.e. 0 ˚C) was measured using a glass rod thermometer (Laboratory Thermometer (Non Mercury) -10 °C to +110 °C, Chem Lab Supplies, South Africa). At the 2-hour mark, the vials were removed from the ice-water slurry. The moths were placed on their backs, individually in 9 cm diameter petri dish bases (on the laboratory bench at 26 ˚C and 72 % RH), and the time taken to recover (i.e. regain muscle control, turn over and stand upright on their legs again) from the cold stress were scored (using a timer) to the nearest second, as chill coma recovery time (McMillan *et al.*, 2012).

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2.4.5 Oviposition testing

2.4.5.1 Male and female mating frequency

To determine mating frequency of the males emerging from each diet, a freshly emerged virgin male and female moth pair was placed into a 500 ml paper drinking cup, containing a pleated cardboard oviposition substrate (50 x 10 mm when pleated five times) held together with a paper clip. A 10 mm dental cotton wick soaked with distilled water for adults to drink from, was attached to the paper cup lids (Walton and Conlong, 2016). The lids were placed on the paper cups after the moth pair and oviposition substrate was placed in it, and labelled accordingly (i.e. diet formulation, harvested date, inoculated date). The paper cups were placed upright in plastic storage baskets and placed on 5-tier metal racks in the IRU adult emergence and oviposition room (maintained at 26 ± 2 °C, 72 ± 5 % RH and 8-hour L: 16-hour D photo phase).

On a daily basis, the oviposition substrate and the female moth was removed and the water supply was replenished. A new oviposition substrate and a freshly emerged female was placed into the paper cup with the remaining male and left to mate overnight (Walton and Conlong, 2016). The oviposition substrate was not used for fecundity in this section but was placed in the cup to prevent the females from randomly laying eggs inside the cups. The female removed was killed by freezing and placed into a plastic resealable bag labelled with the male she was paired with and the date she was placed with the male. The female was then dissected under a light dissecting microscope to assess mating status by checking her bursa copulatrix for the presence of spermatophores (Walton and Conlong, 2016). The procedure was repeated with all the females presented to each male until the male died, to determine how many females the males from each diet successfully copulated with in its life time. A total of fifteen males per diet formulation were used. A similar procedure was conducted for freshly emerged virgin males imposed to chill coma treatment, mated with non-imposed virgin females.

To determine female mating frequency, pairing of virgin female moths with virgin male moths, from each diet formulation, was prepared as described above. Daily, the oviposition substrate and the male moth was removed and the water supply was replenished. A new oviposition substrate and freshly emerged male moth was placed into the paper cup with the remaining female moth and left to mate overnight (Walton and Conlong, 2016). This procedure was repeated until the female died, after which she was dissected to assess mating frequency by counting the spermatophores within her bursa copulatrix, assuming the males she was paired with transferred only one spermatophore to her bursa copulatrix on the night with her. (Walton and Conlong, 2016). A total of 15 females per diet formulation were used to assess mean mating frequency. A similar procedure was conducted for freshly emerged virgin females imposed to chill coma treatment, mated with non-imposed virgin males.

2.4.5.2 Female fecundity and fertility

To determine female fecundity, a single freshly emerged male and female moth pair (15 moth pairs per diet formulation) was placed into a 500 ml paper drinking cup prepared as described in section 2.4.5.1 above. Daily, oviposition substrates were replaced and the water supply was replenished until the female moth died (Walton and Conlong, 2016). Each collected oviposition substrate was inserted into a plastic resealable bag, labelled accordingly (i.e. diet formulation, inoculated date, cup number, date of removal) and placed in an incubator, kept at 24 °C, 72 % RH and zero L: 24-hour D photophase. To determine mean fecundity per *E. saccharina* female, total number of eggs laid on the oviposition substrates each day were counted under a light dissecting microscope for each female in the trial. These were summed for each female, and divided by the number of females in the trial, to get the mean fecundity of each female in the trial (Walton and Conlong, 2016).

To determine mean fertility of the eggs laid per female, total number of black head stage eggs (Goebel, 2006) or neonate larvae emerging from the eggs laid by each female each day, were

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counted under a light dissecting microscope, five days after each oviposition substrate was removed from the cup, to allow the neonate larvae to develop in the eggs. Unfertilised eggs were also counted. The fertile and infertile eggs laid per day per female were summed, and percentage fertilised eggs were calculated per female. These were then summed for each female and divided by the number of females in the trial, to get the mean fertility of the eggs laid by each fertilised female in the trial (Walton and Conlong, 2016). A similar procedure for determining fecundity and fertility was conducted for females imposed to chill coma treatment, mated with non-imposed virgin males.

2.5 Statistical analysis

One-Way Analysis of variance (ANOVA) was performed using IBM SPSS Statistics version 22 (IBM Corp., 2013), on the diet pH, moisture content and water activity data, to compare means between the different diet formulations listed in Table 2.1. ANOVA was also performed on percentage survival and population age distribution (at the time to first pupation and at full pupal harvest), male and female pupal weight, moth emergence and sex ratio, male and female chill coma recovery time, male and female mating frequency and female fecundity and fertility data, to compare means between the different diet formulations listed in Table 2.1, Table 2.2 and Table 2.3. Significant means were separated with a Tukey's HSD post hoc test, at $P < 0.05$. Key assumptions of ANOVA were checked and met for homogeneity of variance (using the Levene's test, at $P > 0.05$) and normality (using a Shapiro-Wilk test for normality) of data distributions. A two-sample t-test ($p < 0.05$) was performed, using IBM SPSS Statistics version 22, on male and female mating frequency and fecundity and fertility data from the diets listed in Table 2.3, to compare means between chill coma imposed moths and those that were not exposed to the chilling treatment. To test whether the data sets for chill coma imposed and non-imposed moths were normally distributed, a W-test for normality was used. P values less than 0.05 were deemed to be significantly different. The SC and PAP diets described in Table 2.1 did not produce any results and thus were not included in the data analysis.

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Chapter 3. Results

3.1 Physical properties of artificial diets

Table 3.1 Mean (± SE) pH, moisture content and water activity of the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets from the period of inoculation up until harvest (4 - week testing period).

Diet Formulation	Diet pH	Moisture Content (%)	Water Activity (a_w)
MS	4.78 ± 0.04	81.06 ± 3.30	0.90 ± 0.01
IAAP2	4.76 ± 0.04	79.86 ± 2.59	0.92 ± 0.01
IAAP3/4	4.78 ± 0.06	83.34 ± 1.56	0.93 ± 0.01
IAAP5/6	4.80 ± 0.01	82.92 ± 2.57	0.93 ± 0.01
ECBMOD	4.85 ± 0.03	80.00 ± 2.51	0.93 ± 0.01

The pH, moisture content and water activity did not vary between the diet formulations. pH: 4.79 ± 0.02 [n = 4] (p = 0.535), moisture content: 81.43 ± 1.07 % [n = 4] (p = 0.807) and water activity: 0.92 ± 0.00 a_w [n = 4] (p = 0.102) over the 4-week period tested (Table 3.1). No biological contamination was observed in any of the diet formulations throughout the duration of the trial.

3.2 Insect production during quality control and at harvest

3.2.1 Carcass Milling Technique

Table 3.2 Mean (± SE) survival of *Eldana saccharina* life stages reared on the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets from inoculation of neonates to first pupation [n = 15] at day 20 and full pupal production at harvest [n = 85] (Day 27).

Diet Formulation	% Survival at First Pupation (Day 20)	% Survival at Full Pupal Development (Day 27)
MS	100 ± 0.00	99 ± 0.60
IAAP2	100 ± 0.00	99 ± 0.83
IAAP3/4	97 ± 3.33	98 ± 1.31
IAAP5/6	100 ± 0.00	100 ± 0.00
ECBMOD	93 ± 4.54	100 ± 0.00

Survival from neonate to large instar/pupal stage did not vary between the diet formulations. Overall, survival was good, with a mean of 98 ± 1.14 % [n = 15] (p = 0.243) developing in the time to first pupation trial, and a mean of 99 ± 0.33 % [n = 85] (p = 0.370) surviving in the full pupal harvest trial (Table 3.2).

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3.2.2 Diet incorporation of sterols

Table 3.3 Mean (± SE) survival of *Eldana saccharina* life stages reared on the MS (0.1gC), MS (1.0gC), MS (0.1gS), MS (1.0gS), MS (0.2gC:0.2gS), MS (0.5gC:0.5gS) and ECBMOD diets from inoculation of neonates to first pupation [n = 15] at day 20 and full pupal production at harvest [n = 85] (Day 27).

Diet Formulation	% Survival at First Pupation (Day 20)	% Survival at Full Pupal Development (Day 27)
MS (0.1gC)	100 ± 0.00	98 ± 1.01
MS (1.0gC)	100 ± 0.00	100 ± 0.00
MS (0.1gS)	93 ± 4.54	97 ± 1.28
MS (1.0gS)	100 ± 0.00	96 ± 1.40
MS (0.2gC:0.2gS)	100 ± 0.00	99 ± 0.59
MS (0.5gC:0.5gS)	93 ± 4.54	99 ± 0.59
ECBMOD	100 ± 0.00	100 ± 0.00

There were no significant differences observed in survivability of neonates inoculated in all diets. Overall, survival was good, with a mean of 98 ± 0.95 % [n = 15] (p = 0.108) of the neonates developing through to 5th and 6th instar larvae, pre-pupae and pupae in the time to first pupation trial, and a mean of 99 ± 0.33 % [n = 85] (p = 0.065) of these life stages surviving up till full pupal harvest (Table 3.3).

3.2.3 Evaluation of *Eldana saccharina* performance between diets

All *E. saccharina* reared on the different diets tested in the trials performed exceptionally well during development time to first pupation and at the time of full pupal harvest (Table 3.2 and Table 3.3). At 20 days after inoculation more than 92 % of the neonates had survived, and more than 95 % survived up till full pupal harvest.

3.3 Population age distribution

3.3.1 Carcass Milling Technique

Table 3.4 Mean (± SE) distribution of *Eldana saccharina* life stages (percentage of the 1st/2nd instar larvae, 3rd/4th instar larvae, 5th/6th instar larvae, pre-pupae, pupae and mortality) [n = 30] surviving on the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets, 20 days after inoculation. Means within columns with different lower case letters indicate significant differences (p < 0.05).

Diet Formulation	Life Stage Distribution (%)					
	1st/2nd Instar	3rd/4th Instar	5th/6th Instar	Pre-pupae	Pupae	Mortality
MS	2 ± 0.58c	17 ± 0.58c	64 ± 0.58a	5 ± 0.58b	12 ± 0.58b	0 ± 0.00c
IAAP2	4 ± 0.58c	39 ± 0.58a	48 ± 0.58c	0 ± 0.00c	9 ± 0.58c	0 ± 0.00c
IAAP3/4	0 ± 0.00c	17 ± 0.58c	55 ± 0.58b	5 ± 0.58b	20 ± 0.58a	3 ± 0.58b
IAAP5/6	10 ± 0.58b	25 ± 0.58b	50 ± 0.58c	5 ± 0.58b	10 ± 0.33bc	0 ± 0.00c
ECBMOD	15 ± 0.58a	10 ± 0.58d	45 ± 0.58d	10 ± 0.58a	10 ± 0.58bc	10 ± 0.58a

After 20 days, the nutrients supplied by the IAAP3/4 diet and then the MS diet resulted in the fastest development of *E. saccharina* from neonates through to pupae (25 % and 17 % prepupae and pupae, respectively; Table 3.4). This is also evidenced by the lower percentage of larvae in the IAAP3/4 (1st/2nd Instar (0 %), 3rd/4th instar (17 %) and 5th/6th Instar (55 %)) and the MS (1st/2nd Instar (2 %), 3rd/4th instar (17 %) and 5th/6th Instar (64 %)) diets. Even though the ECBMOD diet had a total of 20 % pupae at the same time, the % 5/6 instars (45 %) and also the smaller instar (1st/2nd Instar

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and 3rd/4th instar = 25 %) populations were greater than that in the IAAP3/4 and MS diets. The *E. saccharina* in the other diets tested showed significantly slower growth (Table 3.4).

Table 3.5 Mean (± SE) distribution of *Eldana saccharina* life stages (percentage of larvae, pre-pupae, pupae, moths and mortality) [n = 170] recorded at the time of full pupal harvest (Day 27) on the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets. Means within columns with different lower case letters indicate significant differences ($p < 0.05$).

Diet Formulation	Life Stage Distribution (%)				
	Larvae	Pre-pupae	Pupae	Moths	Mortality
MS	2 ± 0.58c	5 ± 0.58b	83 ± 0.58d	9 ± 0.58a	1 ± 0.58a
IAAP2	5 ± 0.58b	8 ± 0.58a	80 ± 0.58e	6 ± 0.58b	1 ± 0.58a
IAAP3/4	2 ± 0.58c	3 ± 0.58bc	86 ± 0.58c	7 ± 0.58ab	2 ± 0.58a
IAAP5/6	10 ± 0.58a	1 ± 0.58c	89 ± 0.58b	0 ± 0.00c	0 ± 0.00a
ECBMOD	0 ± 0.00c	1 ± 0.58c	99 ± 0.58a	0 ± 0.00c	0 ± 0.00a

After 27 days of development at the same constant temperatures, *E. saccharina* developed fastest in the IAAP3/4 and MS diets (93 % and 92 % pupae and moths, respectively). Even though 99 % of the larvae had pupated in the ECBMOD diet, no moths had emerged yet. *Eldana saccharina* in the other diets developed significantly slower at the same constant temperatures (Table 3.5).

3.3.2 Diet incorporation of sterols

Table 3.6 Mean (± SE) distribution of *Eldana saccharina* life stages (percentage of the 3rd/4th instar larvae, 5th/6th instar larvae, pre-pupae, pupae and mortality) [n = 30] surviving on the MS (0.1gC), MS (1.0gC), MS (0.1gS), MS (1.0gS), MS (0.2gC:0.2gS), MS (0.5gC:0.5gS) and ECBMOD diets, 20 days after inoculation. Means within columns with different lower case letters indicate significant differences ($p < 0.05$).

Diet Formulation	Life Stage Distribution (%)					
	1st/2nd Instar*	3rd/4th Instar	5th/6th Instar	Pre-pupae	Pupae	Mortality
MS (0.1gC)	0 ± 0.00	0 ± 0.00b	27 ± 0.58d	9 ± 0.58b	64 ± 0.58b	0 ± 0.00c
MS (1.0gC)	0 ± 0.00	0 ± 0.00b	30 ± 0.58c	7 ± 0.58bc	63 ± 0.58b	0 ± 0.00c
MS (0.1gS)	0 ± 0.00	0 ± 0.00b	29 ± 0.58cd	2 ± 0.58d	62 ± 0.58b	7 ± 0.58a
MS (1.0gS)	0 ± 0.00	0 ± 0.00b	16 ± 0.58f	12 ± 0.58a	72 ± 0.58a	0 ± 0.00c
MS (0.2gC:0.2gS)	0 ± 0.00	0 ± 0.00b	24 ± 0.58e	6 ± 0.58c	70 ± 0.58a	0 ± 0.00c
MS (0.5gC:0.5gS)	0 ± 0.00	0 ± 0.00b	33 ± 0.58b	7 ± 0.58bc	55 ± 0.58c	5 ± 0.58b
ECBMOD	0 ± 0.00	35 ± 0.58a	50 ± 0.58a	0 ± 0.00d	15 ± 0.58d	0 ± 0.00c

* No available P-value

The incorporation of sterols into the MS diet developed using the carcass milling technique, showed significant effect on *E. saccharina* larval development, irrespective of the type and concentration of sterol added, compared to the control diet (ECBMOD). At 20 days after inoculation, all neonates inoculated onto the MS diets with sterols added, had fast larval development. This is evident by the higher percentage of pupae (≥ 55 %), lower percentage of the 5th/6th instar larvae (≤ 33 %) and no smaller instar (1st/2nd and 3rd/4th instar) larvae, whereas larval development in the ECBMOD diet was much slower producing a higher percentage of the 3rd/4th instar (35 %), 5th/6th instar larvae (50 %) and fewer pupae (15 %) (Table 3.6).

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Table 3.7 Mean (± SE) distribution of *Eldana saccharina* life stages (percentage of larvae, pre-pupae, pupae, moths and total mortality) [n = 170] recorded at the time of full pupal harvest (Day 27) on the MS (0.1gC), MS (1.0gC), MS (0.1gS), MS (1.0gS), MS (0.2gC:0.2gS), MS (0.5gC:0.5gS) and ECBMOD diets. Means within columns with different lower case letters indicate significant differences ($p < 0.05$).

Diet Formulation	Life Stage Distribution (%)				
	Larvae	Pre-pupae	Pupae	Moths	Mortality
MS (0.1gC)	2 ± 0.58c	12 ± 0.58b	82 ± 0.58c	4 ± 0.58a	0 ± 0.00a
MS (1.0gC)	0 ± 0.00c	0 ± 0.00d	96 ± 0.58a	4 ± 0.58a	0 ± 0.00a
MS (0.1gS)	2 ± 0.58c	11 ± 0.58bc	86 ± 0.58b	1 ± 0.58b	0 ± 0.00a
MS (1.0gS)	6 ± 0.58b	9 ± 0.58c	82 ± 0.58c	2 ± 0.58ab	1 ± 0.58a
MS (0.2gC:0.2gS)	6 ± 0.58b	11 ± 0.58bc	82 ± 0.58c	0 ± 0.00b	1 ± 0.58a
MS (0.5gC:0.5gS)	7 ± 0.58b	19 ± 0.58a	73 ± 0.58d	0 ± 0.00b	1 ± 0.58a
ECBMOD	19 ± 0.58a	9 ± 0.58c	70 ± 0.58e	0 ± 0.00b	2 ± 0.58a

The cholesterol containing MS (1.0gC) diet produced the highest proportion of pupae (96 %) at harvest after 27 days, and if % adult emergence (4 %) was added to the % pupation, 100 % of the neonates inoculated had pupated and had some adults emerge from the pupae. In this diet, there were no longer any larvae found. In contrast, only 70 % of the neonates inoculated had pupated by 27 days in the ECBMOD control diet. At this time, 19 % of the life stages in the ECBMOD diet were still larvae, compared to between 0 and 7 % in the sterol containing MS diets. The MS diet containing the higher concentration of the sterol mix (0.5gC:0.5gS) had a similar pupation rate (73 %) to the control, and the lowest pupation for all the sterol containing diets (Table 3.7).

3.3.3 Evaluation of *Eldana saccharina* performance between diets

The IAAP3/4 and MS diets developed using the carcass milling technique produced the highest proportion of 5th to 6th instar larvae and pupae at day 20, and again these diets produce more pupae and moths at harvest (day 27), outperforming the control diet. The control diet produced higher proportions of the smaller instar larvae (1st/2nd Instar and 3rd/4th instar) at day 20 and although this diet produced the highest percentage of pupae at harvest, these had not emerged to moths yet, meaning that development of *E. saccharina* was much slower on this diet compared to the IAAP3/4 and MS diets (Table 3.4 and Table 3.5). When sterols were incorporated into the MS diet, irrespective of the type of sterol added, the spread of larval development in the cholesterol containing diets was narrower than in the carcass milling diets and the controls at day 20 (Table 3.6). The MS diet containing 1.0 g of cholesterol was superior, with all neonates inoculated developing into pupae by day 27, and adult moths emerging. There were no larvae present when this diet treatment was harvested (Table 3.7).

3.4 Male and female pupal weight

3.4.1 Carcass Milling Technique

In all diet formulations, female pupal weight was close to double that of male pupal weight. Male pupal weight was lowest in the IAAP3/4 diet (weight: 0.1045 ± 0.01 g [n = 30]; p = 0.027) compared to the ECBMOD control diet, but not significantly different from the pupae produced in the other diet formulations. Female pupal weight was significantly lowest in the MS (weight: 0.1864 ± 0.01 g [n = 30]; p = 0.021) and IAAP2 (weight: 0.1835 ± 0.01 g [n = 30]; p = 0.041) diets compared to the ECBMOD control diet, but not significantly different from pupae produced in the other diet formulations (Figure 3.1).

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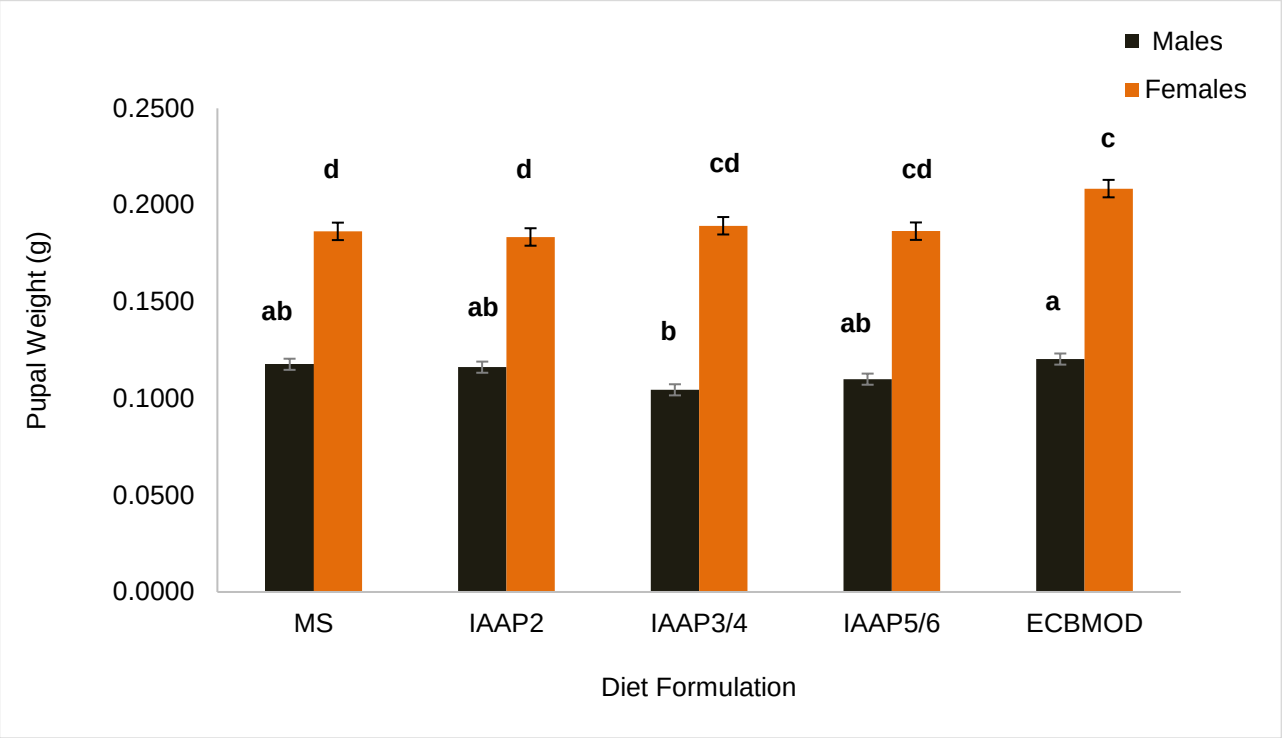

Figure 3.1 Mean (± SE) *Eldana saccharina* male and female pupal weights [n = 30] at harvest (Day 27) for the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets. Different lower case letters above the graph histogram bars indicate that mean differences are significant at the 0.05 level.

3.4.2 Diet incorporation of sterols

As in the carcass milling diets, female pupae were significantly heavier than male pupae. The addition of different sterols and concentrations of sterols did not result in any significant differences in male pupal weights between diet formulations (weight: 0.1199 ± 0.00 g [n = 30]; p = 0.062). However, there were significant differences in female pupal weights as those from the MS (0.1gC) (weight: 0.2143 ± 0.01 g [n = 30]; p = 0.042), MS (0.1gS) (weight: 0.2271 ± 0.01 g [n = 30]; p < 0.001) and MS (0.2gC:0.2gS) (weight: 0.2252 ± 0.01 g [n = 30]; p = 0.001) diets were not significantly heavier than each other, but were significantly heavier than those from the ECBMOD control diet (Figure 3.2).

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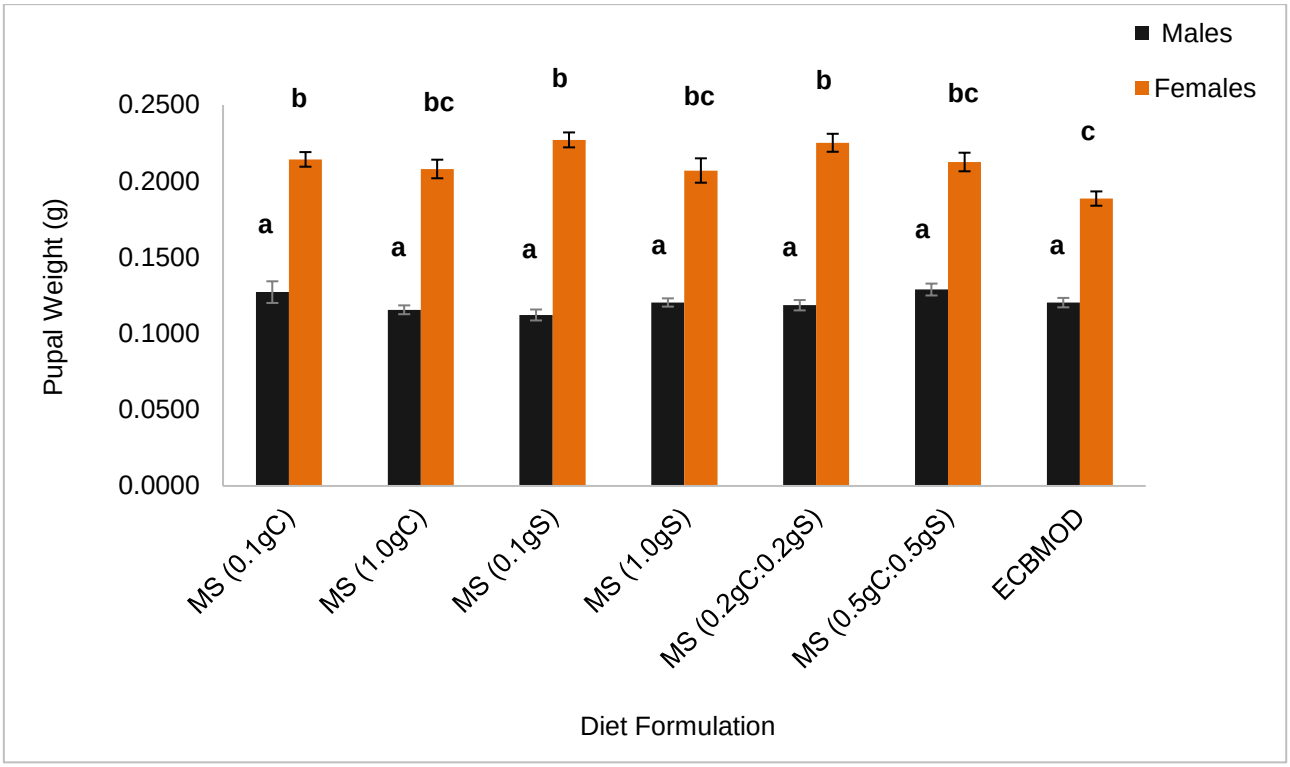

Figure 3.2 Mean (± SE) *Eldana saccharina* male and female pupal weights [n = 30] at harvest (Day 27) for the MS (0.1gC), MS (1.0gC), MS (0.1gS), MS (1.0gS), MS (0.2gC:0.2gS), MS (0.5gC:0.5gS) and ECBMOD diets. Different lower case letters above the graph histogram bars indicate that mean differences are significant at the 0.05 level.

3.4.3 Diet incorporation of cryoprotectants

There were no significant differences observed in male pupal weights produced from the diet formulations (weight: 0.1262 ± 0.00 g [n = 30]; p = 0.054), and these were significantly lighter than female pupae. However, there were significant differences in female pupal weights between diet formulations. Female pupae produced from the MS (0.1gP) (weight: 0.2258 ± 0.01 g [n = 30]; p = 0.002) and MS (0.1gT) (weight: 0.2218 ± 0.01 g [n = 30]; p = 0.009) diets, although not significantly different from each other, were significantly heavier than those from the MS (1.0gT) diet (Figure 3.3).

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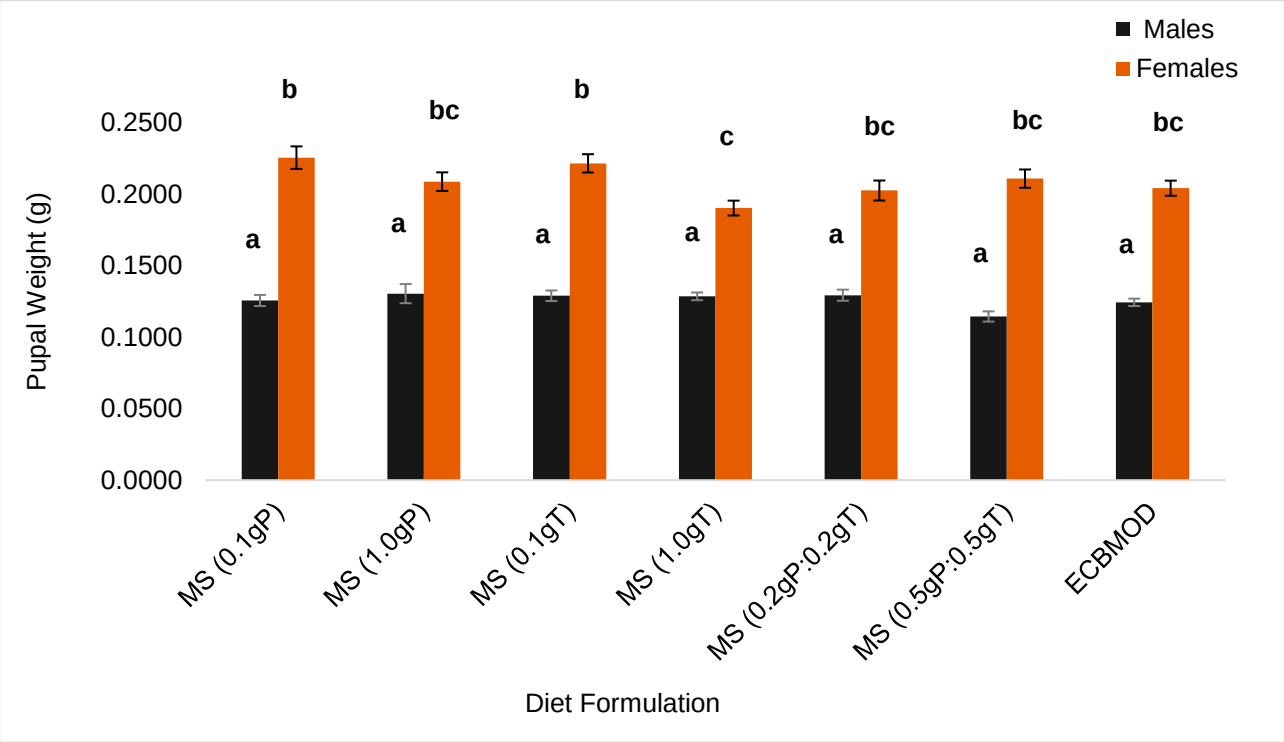

Figure 3.3 Mean (± SE) *Eldana saccharina* male and female pupal weights [n = 30] at harvest (Day 27) for the MS (0.1gP), MS (1.0gP), MS (0.1gT), MS (1.0gT), MS (0.2gP:0.2gT), MS (0.5gP:0.5gT) and ECBMOD diets. Different lower case letters above the graph histogram bars indicate that mean differences are significant at the 0.05 level.

3.4.4 Evaluation of *Eldana saccharina* performance between diets

In all the diets tested in the trials, female pupal weight was close to double that of male pupal weight (Figure 3.1, Figure 3.2 and Figure 3.3). In the carcass milling technique trial (Figure 3.1), male pupal weights were similar to those of the control diet, except for the IAAP3/4 diet, which produced significantly lighter male pupae. Female pupal weights on the MS and IAAP2 diet were lower than that of the control diet. When sterols (Figure 3.2) were incorporated into the MS diet developed using the carcass milling technique, female pupae were heavier than those produced in the carcass milling diets (Figure 3.1). Female pupae from the sterol diets were also heavier than those from the cryoprotectant diets, apart from the MS (0.1gP) and MS (0.1gS) diets, which also produced heavier females (Figure 3.3). Male pupal weights from the cryoprotectant diets (Figure 3.3) were heavier than those produced on the carcass milling diets and the control diet. They were also heavier than those produced from sterol diets, apart from the MS (0.1gC) and MS (0.5gC:0.5gS), which also produced heavier males.

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3.5 Moth emergence and sex ratio

3.5.1 Carcass Milling Technique

Table 3.8 Mean (± SE) emergence and sex ratio (Male: Female) of *Eldana saccharina* moths [n = 130] from pupae harvested from the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets. Means within columns with different lower case letters indicate significant differences ($p < 0.05$).

Diet Formulation	Moth Emergence (%)	Sex Ratio (M:F)
MS	98 ± 0.58a	0.9 ± 0.06
IAAP2	91 ± 0.58b	1.1 ± 0.06
IAAP3/4	97 ± 0.58a	1.1 ± 0.06
IAAP5/6	90 ± 0.58b	1.0 ± 0.06
ECBMOD	96 ± 0.58a	1.1 ± 0.06

Moth emergence from pupae harvested from all diet formulations was high (≥ 90 %). The highest emergence was from pupae harvested from the MS diet (emergence: 98 ± 0.58 % [n = 170]; $p < 0.001$), followed by those from the IAAP3/4 (emergence: 97 ± 0.58 % [n = 170]; $p < 0.001$) and ECBMOD (emergence: 96 ± 0.58 % [n = 170]; $p < 0.001$) diets. The sex ratio of adults emerging from all pupae harvested from the different diet formulations were all close to 1 (ratio: 1.0 ± 0.03 [n = 170]; $p = 0.119$) meaning that the sex ratios are not biased towards males nor females (Table 3.8).

3.5.2 Diet incorporation of sterols

Table 3.9 Mean (± SE) emergence and sex ratio (Male: Female) of *Eldana saccharina* moths [n = 130] from pupae harvested from the MS (0.1gC), MS (1.0gC), MS (0.1gS), MS (1.0gS), MS (0.2gC:0.2gS), MS (0.5gC:0.5gS) and ECBMOD diets. Means within columns with different lower case letters indicate significant differences ($p < 0.05$).

Diet Formulation	Moth Emergence (%)	Sex Ratio (M:F)
MS (0.1gC)	96 ± 0.58b	1.3 ± 0.06a
MS (1.0gC)	96 ± 0.58b	1.1 ± 0.06a
MS (0.1gS)	100 ± 0.33a	0.8 ± 0.06b
MS (1.0gS)	94 ± 0.58c	0.9 ± 0.06b
MS (0.2gC:0.2gS)	100 ± 0.33a	1.2 ± 0.06a
MS (0.5gC:0.5gS)	92 ± 0.58c	1.1 ± 0.06a
ECBMOD	97 ± 0.58b	0.8 ± 0.06b

Moth emergence from pupae harvested from all diet formulations was high (≥ 92 %). The highest emergence (emergence: 100 ± 0.33 % [n = 170]; $p < 0.034$) was from pupae harvested from the MS (0.1gS) and MS (0.2gC:0.2gS) diet formulations, followed by those from pupae from the control (emergence: 97 ± 0.58 % [n = 170]; $p < 0.034$), MS (0.1gC) (emergence: 96 ± 0.58 % [n = 170]; $p < 0.003$) and MS (1.0gC) (emergence: 96 ± 0.58 % [n = 170]; $p < 0.003$) diets. The sex ratio of adults emerging from pupae harvested from the ECBMOD (ratio: 0.8 ± 0.06 [n = 170]; $p < 0.031$), MS (0.1gS) (ratio: 0.8 ± 0.06 [n = 170]; $p < 0.031$) and MS (1.0gS) (ratio: 0.9 ± 0.06 [n = 170]; $p < 0.031$) diets favoured more females than males compared to pupae from the remaining diets (Table 3.9).

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3.5.3 Diet incorporation of cryoprotectants

Table 3.10 Mean (± SE) emergence and sex ratio (Male: Female) of *Eldana saccharina* moths [n = 130] from pupae harvested from the MS (0.1gP), MS (1.0gP), MS (0.1gT), MS (1.0gT), MS (0.2gP:0.2gT), MS (0.5gP:0.5gT) and ECBMOD diets. Means within columns with different lower case letters indicate significant differences ($p < 0.05$).

Diet Formulation	Moth Emergence (%)	Sex Ratio (M:F)
MS (0.1gP)	95 ± 0.58b	0.9 ± 0.06c
MS (1.0gP)	96 ± 0.58b	1.3 ± 0.06b
MS (0.1gT)	89 ± 0.58c	1.3 ± 0.06b
MS (1.0gT)	100 ± 0.33a	1.2 ± 0.06b
MS (0.2gP:0.2gT)	91 ± 0.58c	1.0 ± 0.06bc
MS (0.5gP:0.5gT)	96 ± 0.58b	1.0 ± 0.06bc
ECBMOD	89 ± 0.58c	1.7 ± 0.06a

Moth emergence from pupae from the different cryoprotectant diet formulations was again high (≥ 89 %), with pupae harvested from the MS (1.0gT) (emergence: 100 ± 0.33 % [n = 170]; $p < 0.005$) all emerging as adults, followed by pupae harvested from the MS (1.0gP) (emergence: 96 ± 0.58 % [n = 170]; $p < 0.005$) and MS (0.5gP:0.5gT) diets (emergence: 96 ± 0.58 % [n = 170]; $p < 0.005$) and the MS (0.1gP) (emergence: 95 ± 0.58 % [n = 170]; $p < 0.002$) diet. Apart from the sex ratio of *E. saccharina* moths obtained from pupae harvested from the ECBMOD diet, which produced more males then females (ratio: 1.7 ± 0.06 [n = 170]; $p < 0.003$) and the MS (0.1gP) diet, which produced more females then males (ratio: 0.9 ± 0.06 [n = 170]; $p < 0.003$), the sex ratio of adults emerging from the remaining diet formulations, were all close to 1 (Table 3.10).

3.5.4 Evaluation of *Eldana saccharina* performance between diets

Moth emergence from the harvested pupae in all diets tested in the trials (Table 3.8, Table 3.9 and Table 3.10) was high (≥ 89 %) and the sex ratio of moths emerging from pupae was close to 1, except for the sex ratio of the control diet produced in the cryoprotectant incorporation trial, where more males were produced than females and the MS (0.1gP) diet, which produced more females than males. It is thus apparent that the addition of sterols or cryoprotectants to the MS diet formulation determined from the carcass milling technique had no effect on the emergence of adults from the pupae harvested from all the diet formulations, nor on their sex ratio.

3.6 Chill coma recovery time

3.6.1 Carcass Milling Technique

There were no significant differences in male chill coma recovery time (recovery time: 267.60 ± 17.16 s [n = 30]; $p = 0.298$). However, there were significant differences in female chill coma recovery time. Females from the MS diet took significantly longer to recover from the imposed chill coma (recovery time: 306.60 ± 10.80 s [n = 30]; $p = 0.023$) than females from the IAAP5/6 which seemed to recover the fastest (recovery time: 220.20 ± 10.20 s [n = 30]; $p = 0.023$). Chill coma recovery time of females from the remaining diet formulations was not significantly different from that of females from the MS and IAAP5/6 diets (Figure 3.4).

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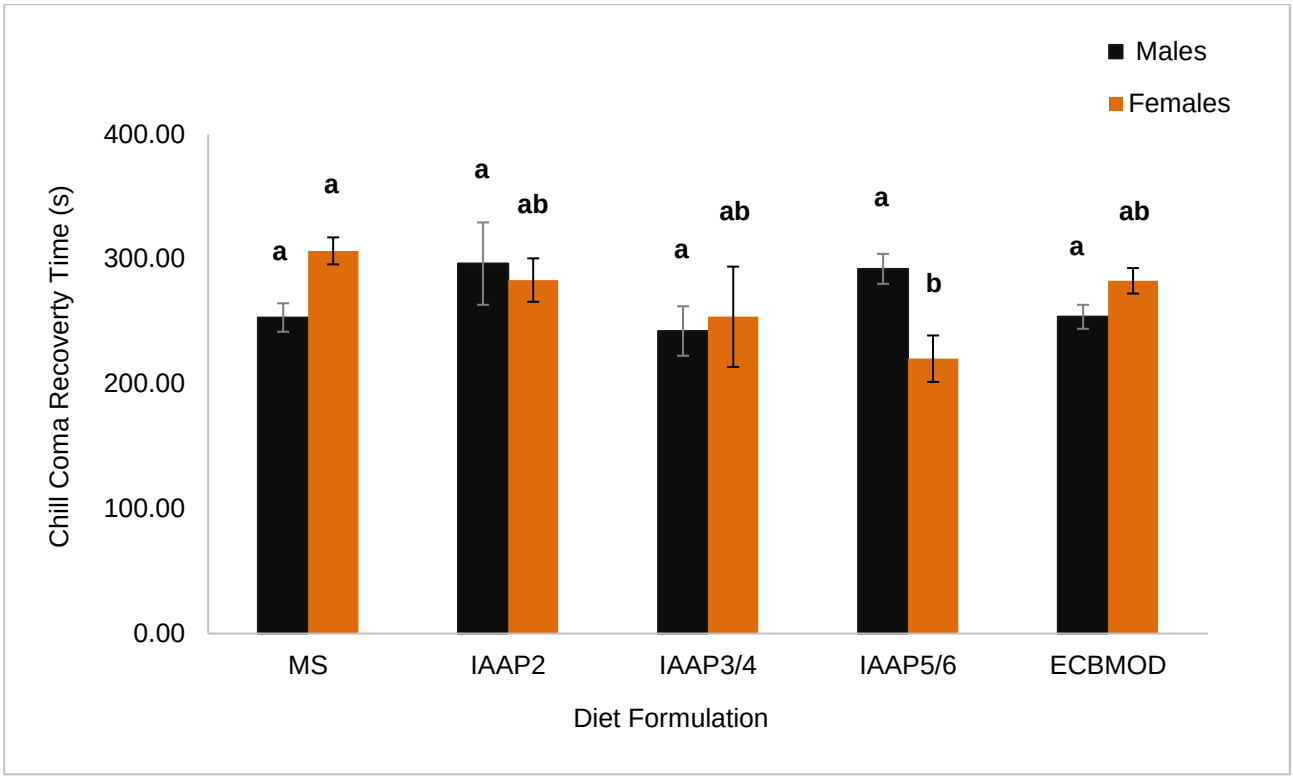

Figure 3.4 Mean (± SE) chill coma recovery time of *Eldana saccharina* male and female moths [n = 30] from the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets. Different lower case letters above the graph histogram bars indicate that mean differences are significant at the 0.05 level.

3.6.2 Diet incorporation of cryoprotectants to enhance chill coma recovery

Females from all formulations had no significant differences in chill coma recovery time, all being around 291.00 ± 36.77 seconds [n = 30] ($p = 0.237$). However, there were significant differences in male chill coma recovery time. Males from the MS (0.2gP:0.2gT), MS (0.5gP:0.5gT) and MS (1.0gT) diets recovered faster from the imposed chill coma (recovery time: 204.00 ± 44.40 s [n = 30]; $p \leq 0.041$, recovery time: 214.80 ± 35.40 s [n = 30]; $p \leq 0.018$ and recovery time: 215.40 ± 7.20 s [n = 30]; $p \leq 0.041$, respectively) than males from the MS (1.0gP), MS (0.1gT) and MS (0.1gP) (recovery time: 294.60 ± 9.00 s [n = 30]; $p = 0.041$, recovery time: 306.60 ± 28.20 s [n = 30]; $p \leq 0.041$ and recovery time: 313.80 ± 29.40 s [n = 30]; $p \leq 0.022$, respectively).

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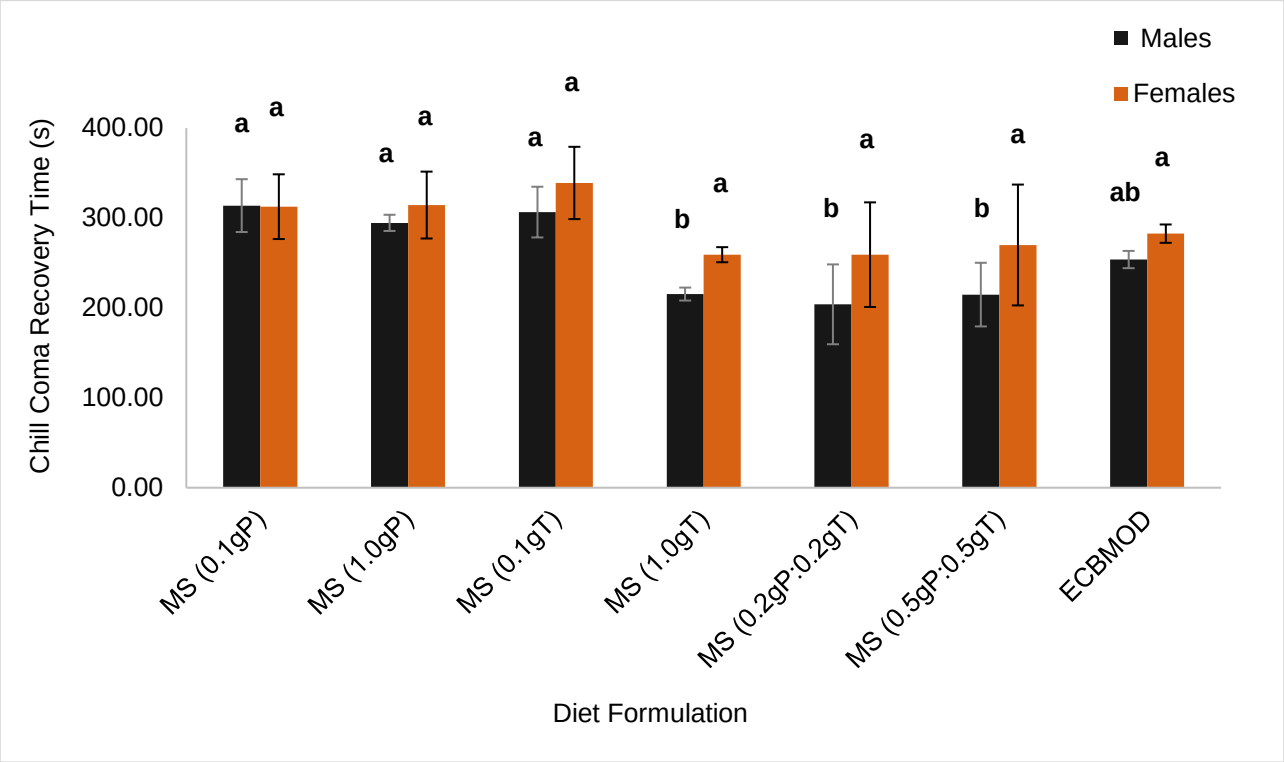

Figure 3.5 Mean (± SE) chill coma recovery time of *Eldana saccharina* male and female moths [n = 30] from the MS (0.1gP), MS (1.0gP), MS (0.1gT), MS (1.0gT), MS (0.2gP:0.2gT), MS (0.5gP:0.5gT) and ECBMOD diets. Different lower case letters above the graph histogram bars indicate that mean differences are significant at the 0.05 level.

3.6.3 Evaluation of *Eldana saccharina* performance between diets

The addition of lower and higher concentrations of the mix of cryoprotectants as well as the addition of trehalose at the highest concentration, enhanced the earlier recovery time from the imposed chill coma in all males coming from the diet formulations, by close to 2 minutes (91 – 110 seconds) compared to the other formulations containing L-Proline (Figure 3.5) and by close to a minute (52 – 64 seconds) compared to all carcass milling formulated diets (Figure 3.4). The addition of cryoprotectants did not seem to have any impact on chill coma recovery of females compared to each other and the carcass milling diets.

3.7 Male and female mating frequency

3.7.1 Carcass Milling Technique

Table 3.11 Mean (± SE) mating frequency of *Eldana saccharina* male and female moths [n = 15] from the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets. Means within columns with different lower case letters indicate significant differences ($p < 0.05$).

Diet Formulation	Male Mating Frequency	Female Mating Frequency
MS	4 ± 0.23b	3 ± 0.19a
IAAP2	5 ± 0.00ab	1 ± 0.00b
IAAP3/4	4 ± 1.76b	1 ± 0.00b
IAAP5/6	6 ± 0.67a	1 ± 0.33b
ECBMOD	4 ± 0.21b	2 ± 0.21ab

The mating frequency of the adult males and females from the different diet formulations varied, with males from the MS, IAAP3/4 and ECBMOD diets mating with the least number of females (mating frequency: 4 ± 0.23 times [n = 15]; p = 0.006, mating frequency: 4 ± 1.76 times [n = 15]; p = 0.041

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and mating frequency: 4 ± 0.21 times [n = 15]; p = 0.012, respectively) and males from the IAAP5/6 diet mating with significantly more females (mating frequency: 6 ± 0.67 times [n = 15]; p ≤ 0.041).

Females from the IAAP2, IAAP 3/4 and IAAP 5/6 diet formulations only mated once (mating frequency: 1 ± 0.00 times [n = 15]; p = 0.021, mating frequency: 1 ± 0.00 times [n = 15]; p = 0.021 and mating frequency: 1 ± 0.33 times [n = 15]; p = 0.017, respectively), despite having the choice to mate with more males, while females from the MS diet formulation mated with significantly more males (mating frequency: 3 ± 0.19 times [n = 15]; p = 0.021) (Table 3.11).

3.7.2 Diet incorporation of cryoprotectants

3.7.2.1 Mating frequency comparisons between chill coma imposed and non-imposed moths from the different diet formulations

Table 3.12 Mean (± SE) mating frequency of chill coma imposed and non-chill coma imposed *Eldana saccharina* male and female moths [n = 15], after being paired with moths from the same formulations but not imposed to chill coma, from the MS (0.1gP), MS (1.0gP), MS (0.1gT), MS (1.0gT), MS (0.2gP:0.2gT), MS (0.5gP:0.5gT) and ECBMOD diets.

Diet Formulation	Male Mating Frequency		Female Mating Frequency	
	Chill Coma Imposed	Non-Chill Imposed	Chill Coma Imposed	Non-Chill Imposed
MS (0.1gP)	3 ± 1.00	3 ± 0.67	3 ± 0.58	2 ± 0.33
MS (1.0gP)	3 ± 0.33	3 ± 0.33	3 ± 0.33	3 ± 0.33
MS (0.1gT)	3 ± 0.67	4 ± 0.33	3 ± 0.33	2 ± 0.33
MS (1.0gT)	4 ± 0.33	4 ± 0.58	2 ± 0.33	2 ± 0.33
MS (0.2gP:0.2gT)	3 ± 0.67	4 ± 0.33	2 ± 0.68	2 ± 0.33
MS (0.5gP:0.5gT)	3 ± 0.58	3 ± 0.33	2 ± 0.6	2 ± 0.33
ECBMOD	3 ± 0.33	4 ± 0.33	2 ± 0.33	2 ± 0.33

Comparisons of mating frequency between moths imposed to chill coma and those that were not imposed to the chilling treatment, from each diet formulation, were made. There were no significant differences observed in mating frequency of males imposed to chill coma and those not imposed to chill coma from each diet formulation (MS (0.1gP): t (28) = -0.277, p = 0.795; MS (1.0gP): t (28) = -1.414, p = 0.230; MS (0.1gT): t (28) = -1.342, p = 0.251; MS (1.0gT): t (28) = -1.000, p = 0.374; MS (0.2gP:0.2gT): t (28) = -0.447, p = 0.678; MS (0.5gP:0.5gT): t (28) = 0.500, p = 0.643; ECBMOD: t (28) = -2.121, p = 0.101) (Table 3.13).

There were no significant differences observed in mating frequency of females imposed to chill coma and those not imposed to chill coma from each diet formulation (MS (0.1gP): t (28) = 1.000, p = 0.374; MS (1.0gP): t (28) = 0.000, p = 1.000; MS (0.1gT): t (28) = 0.707, p = 0.579; MS (1.0gT): t (28) = -1.414, p = 0.230; MS (0.2gP:0.2gT): t (28) = -0.894, p = 0.422; MS (0.5gP:0.5gT): t (28) = -0.894, p = 0.422; ECBMOD: t (28) = 0.001, p = 1.000) (Table 3.13).

3.7.3 Evaluation of *Eldana saccharina* performance between diets

The addition of cryoprotectants to the MS diet seemed to get females from the different diet formulations to mate with at least two or more males, irrespective of being subjected to cold treatment or not (Table 3.12), than females from the carcass milling diet formulations (Table 3.11). The addition of cryoprotectants to the MS diet did not seem to have any impact on male mating ability. Males from the diets containing cryoprotectants were only able to mate with 3 females, irrespective of being subjected to cold treatment or not (Table 3.12), while males from the carcass milling diets were able to mate with 4 or more females (Table 3.11).

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3.8 Female fecundity and fertility

3.8.1 Carcass Milling Technique

Table 3.13 Mean (± SE) fecundity of *Eldana saccharina* female moths [n = 15] and their egg fertility after mating with moths from the same formulations, from the MS, IAAP2, IAAP3/4, IAAP5/6 and ECBMOD diets.

Diet Formulation	Fecundity (n)	Fertility (%)
MS	917 ± 40.06	94 ± 1.65
IAAP2	764 ± 73.14	96 ± 1.17
IAAP3/4	991 ± 105.76	96 ± 2.83
IAAP5/6	963 ± 70.07	94 ± 5.05
ECBMOD	789 ± 48.66	94 ± 1.77

There were no significant differences observed in fecundity (871 ± 28.13 eggs [n = 15]; p = 0.066) and fertility (94 ± 1.01 % [n = 15]; p = 0.783) of female moths produced from the diet formulations when mated with male moths from the same diet formulations (Table 3.14).

3.8.2 Diet incorporation of sterols

Table 3.14 Mean (± SE) fecundity of *Eldana saccharina* female moths [n = 15] and their egg fertility after mating with moths from the same formulations, from the MS (0.1gC), MS (1.0gC), MS (0.1gS), MS (1.0gS), MS (0.2gC:0.2gS), MS (0.5gC:0.5gS) and ECBMOD diets.

Diet Formulation	Fecundity (n)	Fertility (%)
MS (0.1gC)	879 ± 40.32	94 ± 5.65
MS (1.0gC)	955 ± 28.81	96 ± 1.57
MS (0.1gS)	827 ± 90.30	98 ± 2.03
MS (1.0gS)	922 ± 105.06	98 ± 1.34
MS (0.2gC:0.2gS)	891 ± 148.86	91 ± 7.59
MS (0.5gC:0.5gS)	906 ± 20.25	100 ± 0.15
ECBMOD	789 ± 48.66	94 ± 1.77

There were no significant differences observed in fecundity (878 ± 24.44 eggs [n = 15]; p = 0.314) and fertility (96 ± 1.07 % [n = 15]; p = 0.599) of female moths produced from the different diet formulations when mated with male moths from the same diet formulations (Table 3.15).

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3.8.3 Diet incorporation of cryoprotectants

3.8.3.1 Fecundity and fertility comparisons between chill coma imposed and non-imposed female moths from the different diet formulations

Table 3.15 Mean (± SE) fecundity of chill coma imposed and non-chill imposed *Eldana saccharina* female moths [n = 15] and their egg fertility after mating with males from the same formulations but not exposed to chill coma, from the MS (0.1gP), MS (1.0gP), MS (0.1gT), MS (1.0gT), MS (0.2gP:0.2gT), MS (0.5gP:0.5gT) and ECBMOD diets. Means followed by different lower case letters in a line indicate significant differences ($p < 0.05$).

Diet Formulation	Female Fecundity (n)		Female Fertility (%)	
	Chill Coma Imposed	Non-Chill Imposed	Chill Coma Imposed	Non-Chill Imposed
MS (0.1gP)	710 ± 7.22	936 ± 39.50	10 ± 9.68b	90 ± 7.49a
MS (1.0gP)	776 ± 240.01	733 ± 82.50	30 ± 2.00b	95 ± 4.85a
MS (0.1gT)	765 ± 54.58	800 ± 46.00	42 ± 6.28a	78 ± 21.9a
MS (1.0gT)	783 ± 47.61	786 ± 49.19	41 ± 5.99a	80 ± 7.73a
MS (0.2gP:0.2gT)	813 ± 150.82	853 ± 85.50	34 ± 5.82b	95 ± 1.31a
MS (0.5gP:0.5gT)	723 ± 30.84	732 ± 60.50	14 ± 7.59a	64 ± 13.19a
ECBMOD	815 ± 25.42	841 ± 41.15	31 ± 3.72b	88 ± 6.448a

Comparisons of fecundity and fertility between females imposed to chill coma and those that were not imposed to the chill coma and mated with non-chill coma-imposed males from each diet formulation, were made. There were no significant differences observed in fecundity of females imposed to chill coma and those not imposed to chill coma from each diet formulation (MS (0.1gP): $t(28) = -1.701$, $p = 0.224$; MS (1.0gP): $t(28) = 0.659$, $p = 0.553$; MS (0.1gT): $t(28) = -0.180$, $p = 0.870$; MS (1.0gT): $t(28) = -0.034$, $p = 0.976$; MS (0.2gP:0.2gT): $t(28) = -0.289$, $p = 0.793$; MS (0.5gP:0.5gT): $t(28) = -0.052$, $p = 0.963$; ECBMOD: $t(28) = -0.323$, $p = 0.767$) (Table 3.17). However, there were significant differences observed in fertility. Fertility of females, produced from the MS (0.1gP), MS (1.0gP), MS (0.2gP:0.2gT) and ECBMOD diets that were imposed to chill coma, was significantly reduced when compared to the fertility of females from the same formulations that were not imposed to the chilling treatment (MS (0.1gP): $t(28) = -12.562$, $p < 0.001$; MS (1.0gP): $t(28) = -9.105$, $p = 0.001$; MS (0.2gP:0.2gT): $t(28) = -4.620$, $p = 0.033$; ECBMOD: $t(28) = -5.014$, $p = 0.009$) (Table 3.17). It is evident that females exposed to chill coma, once recovered and mated with non-chill coma exposed males, irrespective of cryoprotectant treatment, had the fertility of their eggs severely compromised compared to the fertility of females not exposed to chill coma.

3.8.4 Evaluation of *Eldana saccharina* performance between diets

The addition of sterols into the MS diet formulation did not seem to have any impact on fecundity and fertility of female moths produced from the different formulations. The females produced from these diets laid a similar number of eggs (> 870 eggs) that were more than 90 % fertile (Table 3.14), to that of females produced on the carcass milling diets (Table 3.13). Fecundity of females produced from the diets containing cryoprotectants (Table 3.15), irrespective of the females being subjected to cold treatment or not, was similar to that of females produced by the carcass milling and sterol containing diets. Although fecundity of females produced from the cryoprotectant diets was not affected by the chilling treatment, fertility of chill coma-imposed females, irrespective of the diet formulation they came from, was severely compromised, even though they were mated with males from the same source as the non chill imposed females. It is thus apparent that the addition of sterols and cryoprotectants to the MS diet formulation had no impact on fecundity and fertility of female moths produced from the different diet formulations.

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Chapter 4. General Discussion

A fundamental need of any SIT program is the availability of a consistent and economic supply of good quality insects (Leppla *et al.*, 2009). In Chapter 1 of this thesis, a history of developing artificial diets for SIT programs, and the resultant mass rearing of Lepidoptera in particular which it enabled, is given. Due to the successes of SIT in the control of lepidopteran species, and similarities in the ecology and biology between *T. leucotreta* (which is successfully controlled by an operational SIT program in South Africa) and *E. saccharina*, it is envisioned that a targeted SIT programme against *E. saccharina* could significantly reduce its population in South African sugarcane (Woods *et al.*, 2019[a]). Successful integration of SIT in the *E. saccharina* IPM program at SASRI, however, largely depends on rearing the pest on artificial diet to provide a constant, high quality and large supply of partially sterile male moths, in sufficient numbers needed for effective SIT.

There has been considerable progress in the development of artificial diets for *E. saccharina* since it became a recurring pest in South African sugarcane in the 1980's (Ngomane *et al.*, 2017). Each diet showed improved production and cost benefits over the previous ones (Chapter 1). However, none had been based on carcass milling principles used in animal science to develop feed for livestock and poultry (Babinszky and Barsony, 2013). This approach is used to formulate animal diets based on the animals actual nutrient requirements, and the actual nutrients their natural foods provide (Babinszky and Barsony, 2013).

Recent research demonstrated that this approach could be used to develop high quality, cost effective diets for insects (Woods *et al.*, 2019[a, b, c]). The study reported on in this thesis in particular complemented that done by Woods *et al.* (2019[b]), to develop a cost effective diet that did not compromise on the quality of *E. saccharina* reared on it, using the carcass milling technique. The current diet used to rear *E. saccharina* (Ngomane *et al.*, 2017) was developed from a previously published diet for *O. nubilalis* (Nagy, 1970), which, even though it was much more efficient and more cost effective than the previous diet developed by Walton and Conlong (2016), it specifically did not take into account the actual nutrient requirements needed for good development of *E. saccharina*.

4.1 Are the formulated diets, derived from the carcass milling technique, similar or better at rearing *Eldana saccharina* than the diet of Ngomane *et al.* (2017)?

In terms of the artificial diets developed using the carcass milling technique, their physical properties, as outlined in Chapter 1, all proved as good as the previous diets, and this was reflected on the actual growth of *E. saccharina* on them, which will be expanded on below.

The pH of the diets formulated according to the carcass milling technique and the control diet was found to be between 4.76 and 4.85, with an average of 4.79. Similar results were recorded by Ngomane *et al.* (2017) and Woods *et al.* (2019[b]) who found an average pH of 4.9 and 4.7 in the diets they formulated, respectively. In addition, these reflected the pH of *E. saccharina's* natural host plant, sugarcane (4.5 – 5.5; Chauhan *et. al.*, 2002). This is in accordance with Cohen's (2015) generalisation that insects require a slightly acidic pH range in their diet. Also, moisture content of the carcass milling diets and the control diet was retained between 79.86 and 83.34 % which was slightly higher than the moisture content of sugarcane (68 – 74 %; Hagos *et al.*, 2014). According to Cohen (2015), most plant feeding insects require high water content (between 70 to 90 %) in their diets to sustain their life processes and without the adequate amount of water all life processes fail. In natural foods, water is retained within cell walls and bound to cellular constituents, and in artificial diets a nutritionally inert substance such as agar or carrageenan gel is required to bind water. In the current study, *E. saccharina* larvae did not survive on the SC and PAP natural host plant diets developed using the carcass milling technique and therefore were not reported on. In a similar study conducted by Woods *et al.* (2019[b]) the SC and PAP diets lacked the ability to bind water which supported evaporation, but when moisture content of the diet fell too low, the larvae were unable to

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gain access to nutrients, resulting in poor development and increased mortality of *E.saccharina* feeding on the diet.

Water activity of the carcass milling diets and the control diet was found to be between 0.90 and 0.93 a_w and no biological contamination was observed in all diet formulations. According to Rockland and Nishi (1980), microorganisms (i.e. bacteria, mould, yeast etc.) require free water for growth and every microorganism has a minimum water activity below which it will not grow. At a water activity less than 0.95, micro-organisms are generally inhibited because there is not enough free water available to support pathogen growth (Rockland and Nishi, 1980) and this was evident in the diets formulated in this study, as no contamination was recorded.

In the light of this, the pH, moisture content and water activity of the diets formulated using the carcass milling technique were suitable for the growth of *E. saccharina*. This is further supported by the biological parameters of *E. saccharina* reared on these diets.

4.1.1 Effect of the different diet formulations based on the carcass milling technique, on biological parameters of *Eldana saccharina*

Optimisation of *E. saccharina* mass production for the SIT program at SASRI requires the development of a larval rearing diet that gives high larval survivorship, fast larval development, increased pupal weight, high adult emergence of equal sex ratio and increased fecundity and fertility. The diet should be able to provide high quality adults, in terms of chill coma recovery time and mating capacity for effective field performance when released into the wild population (Sorensen *et al.*, 2012).

4.1.1.1 Insect development

The results obtained from the carcass milling diets (except for the SC and PAP formulations, as already explained) has revealed that *E. saccharina* can develop from neonate larvae to the adult stage with a survival rate of 97-100 % in all diet formulations, indicating that all diet formulations provided the required nutrients for larval development. Feeding the larvae with the MS and IAAP3/4 diets shortened the time to first pupation, from 27 days (Ngomane *et al.*, 2017) to 20 days, compared with larvae fed on the remaining carcass milling diets and the control diet. The duration of the larval development time was reduced using these diets, compared to the larval development times, at the same temperatures, recorded by Gillespie (1993) and Way (1995) on the old sugarcane based diet of Graham and Conlong (1988): 35 and 43 days, respectively. Differences in growth rates between diet formulations may have resulted due to the quality and quantity of available nutrients which also depend on the quality and proportion of ingredients in each formulation (Somda *et al.*, 2017). For most laboratory rearing, larval diets are composed of a mix of ingredients (as demonstrated by the diets developed in this study) and these ingredients are the costliest components of the process (Somda *et al.*, 2017). *Eldana saccharina* mass rearing requires cost-effective production and therefore, mass production would become more affordable and suitable if larval feeding can be minimized, while ensuring that the quality of insects produced remains high. A diet that can shorten the period of larval development will help reduce the costs of the diet, labour and accelerate the production of adults (Epopa *et al.*, 2018). This is important when considering mass rearing *E. saccharina* adults for the SIT program. Rearing *E. saccharina* larvae on the MS and IAAP3/4 diets will help provide more insect generations per year, thus giving more that can be sterilised and released into the field (Epopa *et al.*, 2018).

4.1.1.2 Pupal weight

Within the dietary formulations female pupae were found to be heavier than male pupae, supporting the findings reported by Walton and Conlong (2016), Ngomane *et al.* (2017) and Woods[b] *et al.* (2019). The carcass milling diets did not significantly influence male and female pupal weights compared to the control diet (previously observed by Woods *et al.* 2019[b]) and that reported by

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Ngomane *et al.* (2017). However, male and female pupae produced from these diets were found to be heavier than those produced by the diet developed by Walton and Conlong (2016), proving that the diets developed in this study were of superior quality. The highest pupal weight was recorded on females from the IAAP3/4 diet and males from the MS diet. Female pupal weight is directly proportional to their fecundity and therefore, heavier female pupae will result in bigger adults, providing more eggs laid (Graham, 1990). Large males on the other hand produce large spermatophores. The males prefer mating with large, young and virgin females, and the females prefer mating with large and mid-aged males. Mating of larger parents produces larger offspring (Xu, 2010). Thus, the production of heavier *E. saccharina* females will be beneficial for the SIT program in that, fewer females mated with the larger males can be used to produce high numbers of good quality insects. The production of high quality and heavier partially sterile males would also increase their competitive ability with wild males (Hamden *et al.*, 2013), and thus increase their impact in SIT programs.

4.1.1.3 Moth emergence and sex ratio

The carcass milling diets proved to be of highest quality for rearing *E. saccharina*, resulting in adult emergence rates of 90 to 98 %. The highest emergence came from pupae harvested from the MS diet. This shows an increase of at least 8 % in adult emergence compared to that obtained in the diet used by Walton (2011) who had adult emergence of *E. saccharina* of 89.2 %. Since large scale SIT programs depend on irradiation of mature pupae or freshly emerged adults, to sterilize them before release, it is important to provide an efficient artificial diet for *E. saccharina* larvae that produces enough pupae or moths to supply the overflooding ratios needed for SIT (Parker, 2005).

When mass rearing for releases in control programs and producing insects for experimental purposes, a sex ratio that optimises production of both male offspring (e.g. for SIT) and female offspring (e.g. for mass rearing purposes) is required in the breeding population (Fitz-Earle and Barclay, 1989). In the current study, an even male-to-female ratio of 1:1 in emerged *E. saccharina* moths was observed for all diet formulations, which according to Sampson and Kumar (1985) is slightly different from that found in field populations (42:51).

4.1.1.4 Chill coma recovery time

Multiple factors are known to impact field performance of sterile insects in an SIT programme. These include their biological characteristics, fitness traits and most important, the environmental conditions into which they are released (Boersma, 2018). The natural geographic distribution of *E. saccharina* in South Africa stretches across a wide range of climates, but most predominantly in the subtropical climates found in KwaZulu-Natal and sub-Saharan Africa (Assefa *et al.*, 2006; Kleynhans *et al.*, 2014), where temperatures do not fluctuate widely. In temperate regions in contrast, temperature fluctuates markedly on a daily and seasonal basis, and insects from these regions need to be pre-adapted to frequent and unpredictable thermal variations. They should also be able to recover quickly from chill injuries that may occur during unpredictable cold periods (Colinet *et al.,* 2018). So far, considerable attention is being paid to the capacity of certain insects to survive extreme freezing temperatures, and the induction of antifreeze compounds, or cryoprotectants, that have been identified in many species (Gibert *et al.*, 2000). However, cryoprotection is not the only cold tolerance mechanism, as many species die at temperatures above their freezing point. In this regard, studies have analysed the survival of insects at stressing temperatures far above their freezing point (Gibert *et al.*, 2000; Sinclair *et al.*, 2015).

When insects are exposed to low temperatures, provided that cold treatment is not too long, they enter a reversible cold-induced paralysis driven by a failure of neuromuscular function (Findsen *et al.*, 2014). In this state insects cannot pursue activities that enhance fitness such as feeding, mating or oviposition. Chill coma onset has been associated with the loss of ionic and osmotic homoeostasis during low-temperature exposure (Findsen *et al*., 2014). Recovery from chill coma requires a re-establishment of metabolic homoeostasis, recovery of ionic and water balance and

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repair of critical cellular components (Findsen *et al*., 2014). Insect diet components that include high carbohydrate and protein content are known to help restore metabolic homeostasis and energy balance of cold exposed insects that have fed on these diets, thus increasing their cold tolerance (Andersen *et al.*, 2009; Daniel, 2016). In the current study, the carcass milling diets did not significantly impact chill coma recovery time of male and female moths compared with those fed on the control diet. However, all moths from the different diet formulations recovered from the chill coma treatment in just under 6 minutes. The fastest recovery time was recorded on male moths from the IAAP3/4 diet and females from the IAAP5/6 diet. The results suggest that the diets developed in this study provided an optimal balance of carbohydrates and proteins to help support recovery of *E. saccharina* moths from the chilling treatment.

The *E. saccharina* mass rearing program can, however, benefit from adopting a low temperature treatment as part of their operating procedure, since the program requires storage of sterile insects in cold temperature without loss of performance and to mobilize them quickly upon demand (Colinet *et al.*, 2018). Sterile insects are also exposed to low temperature during shipping from mass-rearing facilities to release sites (Nikolouli *et al.*, 2018). Lastly, temperatures within release sites are different from rearing conditions and may be stressful to released insects, thus implementing thermal conditioning protocols before release may be useful in improving the survival of insects during low temperature exposures (Colinet *et al.*, 2018; Nikolouli *et al.*, 2018).

4.1.1.5 Mating frequency

Efficacy of SIT relies upon released sterile male insects efficiently transferring their sperm, which carries dominant lethal mutations, to wild females (Mudavanhu *et al.*, 2016). Released irradiated females do not play an important role in population suppression when deployed in a program with an Inherited Sterility (IS) component (Vreysen *et al.*, 2016). Thus, success or failure of the technique largely depends on the quality of sterile males and their ability to locate and mate with wild females (Mudavanhu *et al.*, 2016). Understanding mating behaviour of species targeted for SIT, their mating systems and how mass rearing and irradiation impacts them, is one of the most important steps leading to improvements in sterile male performance. This could possibly reduce sterile to wild male overflooding ratios routinely applied to compensate for the lower effectiveness of mass-produced sterile insects (Mudavanhu *et al.*, 2016). Should the released males be as competitive as wild males, rearing costs could be significantly reduced (Walton, 2011).

Walton (2011) reported that the optimum radiation dose that will not compromise the performance of treated *E. saccharina* males in an IS program is 200 Gy. Mudavanhu *et al.* (2016) further investigated mating competitiveness and compatibility of non-irradiated and irradiated *E. saccharina* moths under laboratory and semi-field conditions. They found that there were no mating barriers between released irradiated and non-irradiated populations in their studies. The released irradiated males were able to successfully compete with non-irradiated males for wild females, and they also observed that wild females did not discriminate against irradiated or non-irradiated moths which indicated no negative effects due to laboratory rearing or radiation.

In the current study, each *E. saccharina* male reared from the carcass milling diets and the control diet showed the ability to mate more than once in the laboratory when presented with a freshly emerged virgin female on consecutive nights during their life span. The highest mating frequency was recorded on males reared on the IAAP5/6 diet which mated with an average of 6 different females. Similar observations were made by Walton and Conlong (2016) who reported males mating with an average of 3.3 (maximum of 6) females. The ability of *E. saccharina* males to mate multiple times has important implications for calculating the required release rates of sterilized male moths in order to obtain sufficient sterile to wild male overflooding ratios needed for SIT (Walton, 2011).

When given the opportunity to mate with more than 1 male, females from the carcass milling diets mated with an average of 1.5 males. The highest mating frequency was recorded on females reared on the MS diet which mated with an average of 3 different males. Again, these results were

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in line with those of Walton and Conlong (2016) who reported females mating with an average of 1.5 (maximum of 3) males. The ability of *E. saccharina* females to mate more than once in the laboratory is a good indication for mass rearing purposes in that females reared on the MS diets will be able to mate multiple times with fertile males, producing more offspring. Although females from the MS diets mated multiple times, the majority of females reported in this study mated only once. It is likely that females in the wild will mate once (Atkinson, 1980), and if sterile males successfully mate with wild females, this will reduce the chance of wild females accepting mating with wild males (Walton, 2011).

4.1.1.6 Fecundity and fertility

Mean fecundity and fertility of *E. saccharina* females reared on the carcass milling diets and the control diet were very similar to that of Dick (1945) and Betbeder-Matibet *et al.* (1977) who reported a mean of 750 eggs per female that were 97 % fertile. Fecundity and fertility of *E. saccharina* females obtained in this study were significantly higher than that obtained by Gillespie (1993) who reported an average of 318 eggs per female, reared on the diet of Graham and Conlong (1988) and Walton and Conlong (2016) who reported an average of 518 eggs per female that were 63 % fertile from adults reared on their diet. Ngomane *et al.* (2017) showed that the Walton and Conlong (2016) diet was inferior in terms of quality and insect production to the one based on an *O. nubilalis* diet formulation. On the strength of this, it is clear that the experimental diets developed in the current study were of a superior quality to those produced by Walton and Conlong (2016), resulting in better quality insect being reared from them. The most significant effect that increased fecundity and fertility of *E. saccharina* females would have on an SIT program is the reduction of the costs of mass-rearing as fewer females that produce a lot of fertile eggs will be required to produce more insects that need to be sterilised and released into the field.

The carcass milling technique proved to be effective at developing two high quality diets for mass production of *E. saccharina* and it did not compromise insect growth and development, reproduction and fitness. When the nutritional requirements of *E. saccharina* larvae feeding on the MS and IAAP3/4 diets were closely met, the development period was significantly reduced (17 % and 25 % prepupae and pupae produced at day 20, respectively) compared to the larval development reported by Ngomane *et al.* (2017). When comparing the rest of the biological parameters presented above, the MS and IAAP3/4 diets performed similarly to that of *E. saccharina* reared on the diet of Ngomane *et al.* (2017). This implies that larval rearing on the MS and IAAP3/4 diets at the insectary will accelerate the production of adults, and more good quality insects will be produced throughout the year, leading to a constant and higher individual supply of insects for use in the SIT program against *E. saccharina*.

4.2 Does the incorporation of cholesterol and stigmasterol into the carcass milling derived artificial diet improve *Eldana saccharina's* growth, development and reproduction?

Although the MS and IAAP3/4 diets both provided faster development at day 20, the MS diet resulted in the highest percentage of moths (9 %) during the full pupal harvest trial and resulted in the highest adult emergence (98 %) compared to the IAAP3/4, proving that larval development is faster on the MS diet than the IAAP3/4 diet. Thus, further improvements to the MS diet were investigated through the inclusion of sterols, which have been shown to be essential to insect growth and development (Behmer and Nes, 2003) and have improved insect performance when fed to them in artificial diets (Al-Izzi and Hopkins, 1982; Jing *et al.*, 2013; Babu *et al.*, 2018). The type and concentration of sterols incorporated into the MS diet in the current study were deemed to be optimal with respect to survival, growth and development based on studies with other insects (e.g. Al-Izzi and Hopkins 1982 and Babu *et al.* 2018).

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4.2.1 Effect of the different diet formulations supplemented with different sterol components, on biological parameters of *Eldana saccharina*

4.2.1.1 Insect development

Survival of *E. saccharina* larvae reared on sterol impregnated diets did not differ significantly from that of *E. saccharina* reared on the MS diet without sterols added. However, the larval period was significantly shortened in the sterol diets, irrespective of the type and concentration of sterol added. This is evident by the high percentage of pupae (55 – 72 %) produced at day 20, with the highest percentage of pupae resulting from the MS diet supplemented with a higher concentration (1.0 g) of stigmasterol followed by the diet containing the lower concentration of the sterol mix (0.2 g of cholesterol: 0.2 g of stigmasterol). The MS diet without sterols added produced a higher percentage of large larvae (64 %) and smaller percentage of pupae (12 %) during this time. This reduction in larval development period of *E. saccharina* reared on sterol enriched diets suggests that sterols are required for better and faster development of *E. saccharina*. Sterols can effectively improve the production of this insect and reduce the costs of mass rearing for the SIT program because of the faster larval development time, without impacting on other life cycle traits.

A study conducted by Al-Izzi and Hopkins (1982), found that female larvae developed to pupae more rapidly as concentrations of sitosterol and stigmasterol increased, and increases in cholesterol levels did not affect the larval period, with development not as rapid as with the phytosterols. They also observed that at lower concentrations of sitosterol and stigmasterol, female larvae developed at a significantly slower rate than did males. In the current study, the sexes were not separated, however, similar observations to that of Al-Izzi and Hopkins (1982) were made. At a higher concentration of stigmasterol (1.0 g) *E. saccharina* larval period was significantly reduced compared to larvae from the diets containing cholesterol. The current study also demonstrated that the sterol mix at the lowest concentration (0.2gC:0.2gS) in the diet reduced *E. saccharina's* larval period which is good, and this was similarly demonstrated by Babu *et al.* (2018) who observed pupation rates and emergence to be better on diets containing all sterols (i.e. Cholesterol, Stigmasterol and Sitosterol).

4.2.1.2 Pupal weight

The pupal weights of females fed on the diets incorporated with lower concentrations of cholesterol and stigmasterol (0.1 g) and a combination of the lower mix of sterols (0.2gC:0.2gS) were significantly increased by 0.0279 g, 0.0407 g and 0.0393 g, respectively, compared to the MS diet without sterols added, indicating that sterols are a beneficial addition to diets, especially for the growth of *E. saccharina* females. This has important implications for the SIT program in that fewer females can be used to produce high numbers of good quality insects. In the study conducted by Babu *et al.* (2018), they found that sterol incorporation into their diet increased larval and pupal weights when their study insect fed on it. The incorporation of sterols into the MS diet, irrespective of the type and concentration of sterol added, did not have any effect on male pupal weight, compared to the MS diet without sterols added, suggesting that males may have a smaller sterol requirement than females, and this was similarly demonstrated by Al-Izzi and Hopkins (1982) on males feeding on sterol incorporated diets.

Although sterol incorporation into the MS diet did not have a significant effect on the reproductive parameters, i.e. fecundity and fertility, of *E. saccharina* females, the results in this study demonstrate that the incorporation of sterols into the MS diet improves *E. saccharina's* growth and development. This was evident in the faster development of *E. saccharina* larvae when fed on these diets and the increased female pupal weight compared to the MS diet without sterols added.

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4.3 Does the incorporation of a cryoprotective amino acid, L-proline and a carbohydrate, trehalose into the carcass milling derived artificial diet enhance cold hardiness of *Eldana saccharina* moths?

Changes in temperature greatly influences insect development, growth and behaviour, mainly because insects are ectotherms, meaning that their internal body temperature changes with their environment because they do not generate heat (Chidawanyika and Terblanche, 2011; Terblanche *et al.*, 2017). Low-temperature has been identified to potentially limit insect species distribution (Kleynhans *et al.*, 2014). All insects have a preferred range of temperature at which they will thrive and if the temperature drops below this range, the insects become less active until they eventually cannot move (Sinclair *et al.*, 2003). A gradual decline in temperature, coupled with microclimates caused by daily or seasonal temperature fluctuations, serves to prepare the insect to tolerate freeze temperatures (Sinclair *et al.*, 2003). Such insects respond to temperature changes by continuously adjusting their physiology and behaviour in order to survive and optimize their individual fitness in their environment (Lee and Denlinger, 1991).

In the case of laboratory reared insects, field performance may be compromised, especially when rearing is intended for field releases, due to laboratory adaptation, inbreeding depression, unintended selection or through direct rearing effects (e.g. crowding and artificial diet) (Sorensen *et al.*, 2012; Hoffmann and Ross, 2018). Therefore, when rearing insects for field releases it is important to establish quality control or 'filtering' for specific trait(s) to aid and/or enhance effective field performance or fitness traits of these laboratory reared insects (Chidawanyika and Terblanche, 2011; Sorensen *et al.*, 2012). One way that laboratory reared insects can survive cold conditions not commonly encountered in their rearing environment, is through the accumulation of cryoprotectants, such as sugars and amino acids, that increase their cold tolerance by lowering the freezing point of liquids in the haemolymph (Kostal *et al.*, 2011). To date, a handful of studies have focused on the ability to artificially improve the cold tolerance of an insect that is freeze intolerant and very few studies have attempted to improve the cold tolerance of insects for control by means of SIT (Kostal *et al.*, 2011, Daniel, 2016). In addition to the necessity to improve the cold tolerance of insects for SIT, it is important to consider how cryoprotectants may affect other aspects of the insect's physiology.

In the current study, cryoprotectants were incorporated into the MS diet to investigate whether they improved cold hardiness of *E. saccharina* moths that fed on the diet as larvae, for effective field releases. The types and concentrations of cryoprotectants selected in the current study were deemed to be optimal with respect to egg production and viability, flight ability and cold tolerance based on studies with other insects (e.g. Daniel 2016).

4.3.1 Effect of the different diet formulations incorporated with different cryoprotectant components, on biological parameters of *Eldana saccharina*

The results in this study demonstrated that the incorporation of cryoprotectants into the MS diet improved *E. saccharina's* growth in terms of pupal weight and enhanced its cold hardiness in terms of chill coma recovery time. The diet that gave the best results, outperforming the MS diet without cryoprotectants added, was the diet containing the lower concentration of the cryoprotectant mix (0.2 g of L-proline (P): 0.2 g of trehalose (T), resulting in increased male pupal weight and reduced chill coma recovery time of *E. saccharina* male and female moths on this formulation. Rearing *E. saccharina* on the MS (0.2gP:0.2gT) has important implications for the SIT program in that sterile males will be able to recover faster from chilling temperatures if encountered in release sites. The chilling treatment did not affect mating frequency of *E. saccharina* males and females reared on cryoprotectant diets. This again, has important implications for the SIT program in that the released males imposed to chilling temperatures will be able to mate successfully with wild females after they have recovered from chill coma exposure (Nikolouli *et al.*, 2018). The incorporation of the cryoprotectants to the MS diet provided even fitter males than the previous MS diet without

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cryoprotectants added. This will increase their impact on SIT programs in that high quality and heavier partially sterile males, that have the ability to recover quickly under cold stress and continue mating, will be produced. This will make them more competitive with wild males when released into the field (Hamden *et al.*, 2013).

4.3.1.1 Fecundity and fertility

The cryoprotectant diets did not have any effect on fecundity of *E. saccharina* females, irrespective of being subjected to cold treatment or not, and the fertility of females not imposed to the chilling treatment. The fecundity and fertility of females not imposed to chill coma treatment was found to be similar to that of the of the carcass milling diets, the control diet and the sterol containing diets, proving that these diets are of similar quality and are suitable for rearing *E. saccharina*. However, fertility of chill coma imposed females seemed to be severely compromised, resulting in reduced fertility (≤ 43 %) when compared to the carcass milling, control and sterol diets, when mated with males not exposed to chill coma conditions. According to Findsen *et al*. (2014) and Mensch *et al*. (2017), long-term exposure to low temperatures during adult maturation might decrease fertility after cold recovery as a result of carry-over effects on reproductive tissues. Exposure at 0 ºC for 2 hours may have caused this type of injury to the females, thus affecting overall fertility of *E. saccharina* reared on these diets. The reduced fertility of cold imposed *E. saccharina* females has important implications in terms of SIT, because one of the major concerns in the SIT program is that laboratory reared females may compete with wild females to mate with the F1 adults released (Mudavanhu *et al.*, 2016). If this did occur, the cold chilled females, even if fertile, would be only minimally competitive, thus decreasing the chances of them mating with the released sterile males.

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Chapter 5. Conclusion and Recommendations

Success of the Sterile Insect Technique, as part of an in Integrated Pest Management programme against economically important insect pests, largely depends on the ability to mass produce high quality insect species and establish their colonies under controlled laboratory conditions (Leppla *et al.,* 2009). A rearing system has been developed for mass production of *E. saccharina* at the South African Sugarcane Research Institute. Although mass production of this insect has been set in place, improvements of rearing procedures in terms of developing more cost effective diets for mass rearing purposes, improving the quality of the artificial diets for better production of good quality insects and evaluating insect quality parameters that aid and/or enhance effective field performance, is necessary to optimize *E. saccharina* production in this facility. This will enable constant and large supply of good quality insects needed for effective field releases in the SIT program.

In the current study, three experiments were conducted to evaluate nutritional requirements and formulate better diets for mass rearing *E. saccharina* using the carcass milling technique. The results demonstrated that *E. saccharina* performs better when reared on the minimum specification (MS) diet and the diet that resembles the ideal amino acid profile of the 3rd – 4th instar larvae (IAAP3/4). This was evident from the finding that by feeding *E. saccharina* larvae with the MS and IAAP3/4 diets, shortened their larval development period from 27 days to 20 days compared to the control diet. Even though both the MS and IAAP3/4 diets provided faster development at day 20, the MS diet resulted in the highest percentage of moths (9 %) during the full pupal harvest trial and a higher adult emergence (98 %) than the IAAP3/4 diet, proving that larval development is faster on this diet than the IAAP3/4 diet.

Inclusion of sterols and cryoprotectants into the MS diet were further investigated on their impact on the biological parameters of this insect. When the lower concentration of the sterol mix (0.2 g of cholesterol: 0.2 g of stigmasterol) was incorporated into the MS diet, it exhibited significant effects on *E. saccharina's* growth and development. This was evident from the finding that by feeding *E. saccharina* larvae on this diet reduced its developmental time even further (70 % pupae produced at day 20) compared to the MS diet without sterols added (12 % pupae produced at day 20). Reduction in the development period will accelerate insect production in the insectary thus providing more insect generations per year that can be sterilised and released into the field.

The addition of cryoprotectants into the MS diet, particularly at a concentration mix of 0.2 g L-proline and 0.2 g trehalose, improved *E. saccharina's* growth in terms of pupal weight and enhanced its cold hardiness in terms of chill coma recovery time. This was evident from the finding that by feeding *E. saccharina* larvae on this diet increased male pupal weight by 0.0118 g and reduced chill coma recovery time of *E. saccharina* male and female moths by 49.2 s and 47.4 s, respectively, when compared to that of the MS diet without cryoprotectants added. Production of high quality and heavier males that recover quickly from cold stress and continue mating, will increase their mating competitiveness with wild males when released into the field.

Although fecundity of *E. saccharina* females reared on the cryoprotectant diets was not affected by the chill coma treatment, their fertility was severely compromised resulting in reduced fertility of less than 44 % compared to the carcass milling, control and sterol diets. Reduction in fertility of chill coma imposed females will decrease their mating competitiveness with wild females thus decreasing the chances of them mating with the released sterile males.

Based on these findings, it clear that the MS and IAAP3/4 diets most closely represented the nutrient requirements of *E. saccharina*, leading to its faster development on these formulations. The addition of sterols into the MS diet further improved growth and development of *E. saccharina* larvae, resulting in a much faster larval development period than the previous MS and IAAP3/4 diets. The incorporation of cryoprotectants into the MS diet mostly contributed to improving cold hardiness of *E. saccharina* moths in terms of chill coma recovery time, and had no effect on improving the fertility

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of chill imposed female moths. Although the MS (0.2gP:0.2gT) diet also imposed favourable rearing characteristic for *E. saccharina* larvae fed on it, the MS (0.2gC:0.2gS) is the preferred choice to replace the current diet used to rear *E. saccharina* at SASRI, as it reduced the larval growth period considerably compared to the other diets in this study, including the cryoprotectant ones, without having adverse effects on key quality parameters of *E. saccharina*.

Two important future research foci have been highlighted from this study. These are:

- To investigate the addition of the cryoprotectant mix (0.2gP and 0.2gT) into the sterol diet recommended in this study i.e. MS (0.2gC:0.2gS), and determine how this new diet with both sterols and cryoprotectants impacts the biological parameters of *E. saccharina* larvae feeding on them.

- To look into testing the impact of compromised *E. saccharina* female fertility, as a result of chill coma treatment, on the released sterile males in an SIT program, could it be beneficial?

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